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Mitochondrial Proton Leak and Uncoupling Protein Expression in Transgenic Mice and Human Obesity

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ABSTRACT

The significance of the uncoupling protein-1 (UCP1) in brown adipose tissue (BAT) thermogenesis has been well defined However, the absence of significant amounts of BAT in adult humans, and the desire to reveal the mechanisms modulating skeletal muscle thermogenesis, led to the search for other proteins homologous to UCP1 which could also mediate thermogenesis by uncoupling oxidative phosphorylation. This search led to the discovery of Ucp2 and Ucp3 genes, as well as Ucp4 and brain mitochondrial carrier protein-1 (BMCP1). Ucp3 is of particular interest as a potential mediator of thermogenesis because it is selectively expressed at moderately high levels in human skeletal muscle, a tissue that contributes significantly to resting energy expenditure. In this thesis we attempt to elucidate the true physiological role of the UCP1 homologues, and of UCP3 specifically, by examining mitochondrial proton leak and UCP3 protein expression in: (1) mice that do not express Ucp3 (*Ucp3*-knockout mice), (2) transgenic mice that overexpress human Ucp3 in their skeletal muscle (UCP-3Tg mice), (3) and finally in a distinct population of women who differ in their rate of weight loss.

Our studies with the *Ucp3*-knockout mice show that the mitochondrial proton conductance is reduced in skeletal muscle mitochondria when UCP3 is absent. This result supports the hypothesis that UCP3 is a significant contributor to proton leak in skeletal muscle, and that the remaining UCPs in muscle (*e.g.*, UCP2) do not compensate for the loss of UCP3. The effects of known fatty acid stimulators of UCP1-mediated proton transport (*e.g.*, lauric and linoleic acid) on mitochondrial proton leak kinetics in skeletal muscle mitochondria of *Ucp3*-knockout and controls were also examined. Our results show that in control mitochondria, both lauric and linoleic acid stimulate oxygen consumption and decrease state 4 protonmotive force. In the absence of UCP3, we did not observe any significant effects of the fatty acids, indicating that fatty acids effects may be mediated by UCP3.

Mice overexpressing human UCP3 in skeletal muscle have total Ucp3 mRNA expression that is 66-fold greater than normal, and despite an increase in food intake, they are more lean than controls. By examining the overall kinetics of skeletal muscle mitochondrial proton leak reactions, we have shown that proton conductance is significantly increased in mitochondria from UCP3-Tg mice. This provides support for the hypothesis that UCP3 is somehow involved in mediating proton leak in skeletal muscle mitochondria.

Our results from human studies have provided the first ever measurements of proton leak in human muscle mitochondria. We examined the role of UCP3 and mitochondrial proton leak in patients on an energy restricted diet (900 kcal/day) who were identified as either diet-responsive or diet-resistant. Mitochondria were isolated from biopsy samples removed form the rectus femoris region of the quadricep, and mitochondrial proton leak determinations were carried out. Our results indicate that the state 4 oxygen consumption is 51% higher in the diet-responsive subjects compared to the diet-resistant ones. In addition, Ucp3 mRNA expression was found to be 25% greater in skeletal muscle of diet-responsive compared to diet-resistant individuals, suggesting that the upregulation of this protein could be responsible for the differences seen in the rate of weight loss. The observed differences in leak between these two groups of individuals, and the increase in Ucp3 mRNA in the diet-responsive group suggests that the mitochondrial energetics in human muscle mitochondria may be a potential regulator/predictor of energy metabolism and thus result in one's ability to gain/lose weight.

DEDICATION

This thesis is dedicated to my parents. My parents have made huge sacrifices in their lives so that their children could have a good life, and they were successful. Dad, you are the smartest man I know. Mom, you are always looking past your own needs so you can fulfill ours, you are a beautiful and great woman.

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how I could get through the last two years without you (and your other personality, Chris Rock). You have always made me feel so grand, even if I came to work upset, I never left that way. You will be always be successful in your life because of your kindheartedness and honesty.

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I leave my last thank you for the man in my life. Richard, you are the light at the end of the tunnel. You don't do anything if you don't love it, and I love that

about you. Your dedication to your work, and more importantly to your life, leaves me in awe. Your laughter overjoys me. Your intelligence maddens me. Your love sustains me. Nine years have passed, and my heart still bursts when I see you, even when it's more than twice a month! I look forward to being partners with you in this game called life.

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List of Abbreviations

a_c	cytoplasmic and nuclear $TPMP^+$ activity coefficient
a_c	extracellular $TPMP^+$ activity coefficient
a_m	mitochondrial $TPMP^+$ activity coefficient
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ANOVA	analysis of variance
BAT	brown adipose tissue
BMI	body mass index
BMR	basal metabolic rate
BSA	bovine serum albumin
BWt	body weight
$CaCl_2$	calcium chloride
CO_2	carbon dioxide
CoA	coenzyme A
CPT1	carnitine palmitoyl transferase 1
$CuSO_4 \cdot 5H20$	copper sulphate pentahydrate
ddH20	deionized distilled water
DIT	diet-induced thermogenesis
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis tetraacetic acid
EWAT	epididymal white adipose tissue

$FADH_2$	flavin adenine nucleotide dinucleotide (reduced form)
f.c.	final concentration
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FFA	free fatty acid
FG	fast-twitch glycolytic (muscle fibres)
FOG	fast-twitch oxidative-glycolytic (muscle fibres)

g	centrifugal force
g	gram
GDP	guanosine diphosphate
GTP	guanosine triphosphate

h	hour
H_2O_2	hydrogen peroxide
H&E	haematoxylin and eosin
HCl	hydrochloric acid
HEPES	hydroxyethyl piperazine ethane sulphonic acid
hUcp	human uncoupling protein gene, or it's mRNA transcript
hUCP	human uncoupling protein

InWAT	inguinal white adipose tissue
IgG	immunoglobulin G

$K_2C_4H_4O_6$	potassium tartrate
K_2HPO_4	dipotassium phosphate
KCl	potassium chloride

kDa	kilodalton
KH_2PO_4	potassium dihydrogen orthophosphate
KI	potassium iodide
KOH	potassium hydroxide

L	litre
LCAD	long-chain acyl-CoA dehydrogenase
LiCl	lithium chloride
LiOH	lithium hydroxide
log	logarithm (base 10)
LPL	lipoprotein lipase

M	moles/litre
MCAD	medium chain acyl-CoA dehydrogenase
mg	milligram
$MgSO_4 \cdot 7H_20$	magnesium sulphate
MIM	mitochondrial inner membrane
min	minute
ml	millilitre
mm	millimetre
mM	millimole/litre
mo.	month
mole	mole
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
mV	millivolt
MTE-1	mitochondrial thioesterase-1
MV	mitochondrial matrix volume

Na_2EDTA	ethylenediamine tetraacetic acid (disodium salt)
Na_2HPO_4	disodium hydrogen orthophosphate
NaCl	sodium chloride
$NADH_2$	nicotinamide adenine dinucleotide (reduced form)
NCM	nitrocellulose membrane
NEFA	non-esterified free fatty acid

ng	nanogram
$NiCl_2$	nickel chloride
NIDDM	non-insulin dependent diabetes
nm	nanometre

O	monoatomic oxygen
O_2	molecular oxygen

Pi	inorganic phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
PPAR	peroxisome proliferator ****

RbCl	rubidium chloride
RER	respiratory exchange ratio
RMR	resting metabolic rate
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RQ	respiratory quotient

SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SLB	sample loading buffer
SMR	standard metabolic rate
SNP	single nucleotide polymorphism
SO	slow twitch oxidative (muscle fibres)
State 3	maximal phosphorylating respiration mitochondrial
State 4	maximal non-phosphorylating mitochondrial respiration

TBS	tris-buffered saline
TCA	tricarboxylic acid cycle
TEF	thermic effect of food
$TPMP^+$	methyltriphenylphosphonium cation
TPMP-Br	methyltriphenylphosphonium bromide
Tris	2-amino 2-hydroxy-methylpropane-1,3-diol
Tween-20	polyoxyethylene sorbitan monolaurate

Ucp	uncoupling protein gene, or its mRNA transcript
Ucp3-knockout	mice lacking UCP3
UCP	uncoupling protein
UCP-3Tg	mice overexpressing UCP3

V	volts
v/v	volume per volume

w/v	weight per volume
WAT	white adipose tissue

~	approximately
$\Delta\Psi_m$	mitochondrial membrane potential
Δp	mitochondrial protonmotive force
ΔpH	pH gradient
°C	degrees Celsius
µg	microgram

µL	microlitre
µm	micrometre
µM	micromole/litre
µCi	microcurie

1. INTRODUCTION

1.1 OBESITY AND ASSOCIATED HEALTH RISKS

Obesity is a polygenic disorder with complex etiologies characterized by excessive accumulation of adipose tissue. Obesity is a result of long-term energy intake greater than metabolic expenditure and is increasing in incidence at an alarming rate (Rosenbaum *et al.*, 1997), and has been associated with the development of the most prevalent diseases of modern society. It is the most prominent risk factor for non-insulin-dependent diabetes mellitus (NIDDM), and influences a number of cardiovascular risk factors including hypertension, impaired glycaemic control, and dyslipidemia (Jung, 1997; Kissebah *et al.*, 1989; Pi-Sunyer, 1993; Rosenbaum *et al.*, 1997). Obesity also complicates the management of other disorders such as sleep apnea, infertility, osteoarthritis and chronic obstructive pulmonary disorder (COPD), and many others (Jung, 1997; Rosenbaum *et al.*, 1997). It can be argued that obesity can be classified as the major nutritional disease of the Westernized world. About 30% of adult Canadians, aged 18 to 75, are at increased risk of disability, disease and premature death because they are overweight. In Canada alone, the annual price tag for the direct cost of obesity is about $2 billion (Birmingham *et al.*, 1999). This is cause for national concern since the cost represents 2.4% of the $76.6 billion spend on all diseases in Canada in 1997 (Birmingham *et al.*, 1999).

1.2. ENERGY BALANCE: INTAKE VS. EXPENDITURE

As mentioned, obesity is a disorder of energy balance in which intake chronically

exceeds expenditure. A state of energy balance exists when an animal's dietary energy intake equals its energy expenditure. The control of energy balance in humans is intricate, and as is the case for many regulated physiological systems, the central nervous system plays an integral role.

Control of energy intake is complex and involves multiple neural circuits with specific neuropeptides, neurotransmitters and their cognate receptors. Although in recent years there have been significant advances in understanding the regulation of food intake, research is now being shifted towards understanding the regulation and control of, and the molecular basis for, energy expenditure.

Overall energy expenditure (*i.e.*, heat production or thermogenesis) is the sum of two processes, obligatory and facultative energy expenditure. Obligatory energy expenditure is that which occurs in all organs of the body and includes the energy expenditure essential for cellular functions and processes (*i.e.*, maintenance of ion gradients, protein synthesis, and cardiorespiratory activities; collectively termed as the ATP-consuming processes) and energy expenditure for endothermy (Himms-Hagen, 1990). When an individual is at rest and not digesting food, the heat that is being produced corresponds to the basal metabolic rate (BMR) when measured at thermoneutrality, a temperature which elicits no thermoregulatory effect (Himms-Hagen, 1990). To examine postprandial energy expenditure, it is also necessary to take into consideration the thermic effect of food (TEF) which occurs after feeding in the liver, intestine, white adipose tissue (WAT), and skeletal muscle (Himms-Hagen, 1990). TEF is defined as the energy expenditure required to process food during ingestion, digestion, absorption and storage, and is a significant component of obligatory

thermogenesis. The size of this component may vary according to the composition of the food and the nutritional status of the animal (Himms-Hagen, 1990). One interesting newly studied phenomenon is the energy expenditure of fidgeting-like activities, referred to as non-exercise activity thermogenesis (NEAT) (Levine *et al.*, 1999). Non-exercise activities are the activities of daily living other than exercise and including sitting, standing, walking, and fidgeting. It has been shown that changes in NEAT mediate resistance to weight gain with overfeeding in sedentary adults (Levine *et al.*, 1999). What the components of NEAT are and how their mechanisms are modulated remains to be described.

Facultative thermogenesis accompanies metabolic processes which can be switched on of off by the nervous system. Whereas obligatory thermogenesis occurs in all organs of the body, facultative thermogenesis takes place principally in only two organs, skeletal muscle and brown adipose tissue (BAT). Facultative thermogenesis is controlled in skeletal muscle mainly by the motor nerves and in BAT by sympathetic nerves (Himms-Hagen, 1990). It includes heat production by working muscles (exercise-induced thermogenesis) as well as heat production as a result of thermoregulatory processes such as cold-induced shivering in muscles and cold-induced non-shivering in BAT. Facultative energy expenditure was shown by Rothwell and Stock (Rothwell and Stock, 1979) to also include diet-induced thermogenesis (DIT) in BAT in response to overfeeding. This phenomenon causes metabolic rate to increase, thereby reducing metabolic efficiency and attenuating the expected increase in body energy stores and the development of obesity.

On a cellular level, a large proportion of energy expenditure is used to drive the synthesis of ATP in mitochondria. Most of the ATP is synthesized via oxidative

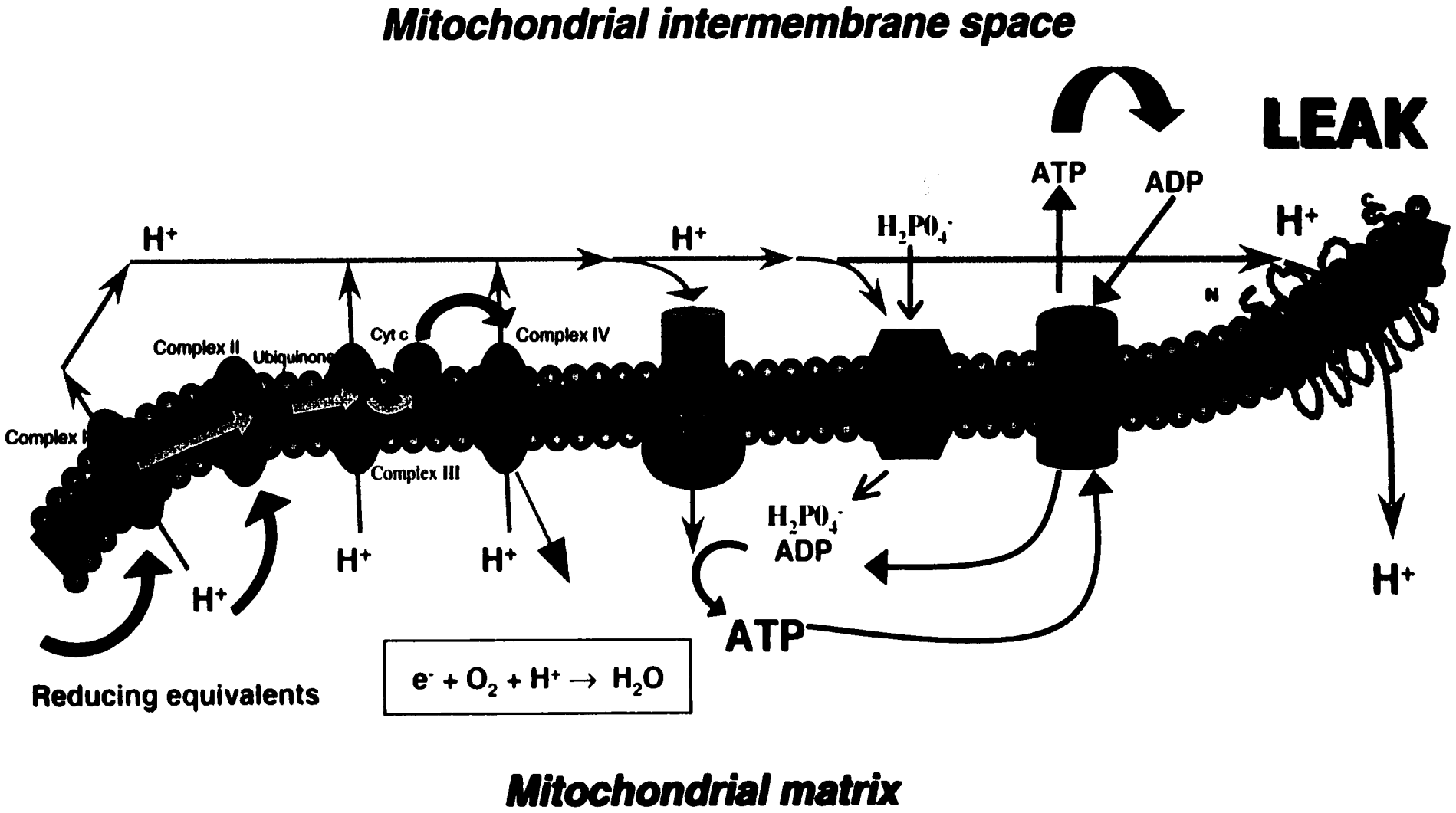
phosphorylation, and the remainder is provided through glycolysis and the tricarboxylic acid cycle. With the main energy equivalent for cellular work being ATP, any mechanism that transfers energy to heat without ATP synthesis is considered to *lower* metabolic efficiency. One such mechanism of heat production is that of 'uncoupling' (*i.e.*, uncoupling substrate oxidation from ATP synthesis), and is known to take place in BAT mitochondria and is mediated by a protein referred to as uncoupling protein-1 (UCP1) (Himms-Hagen, 1990; Nicholls and Locke, 1984). UCP1 carries out its physiological role by facilitating a regulated re-entry of protons into the mitochondrial matrix (mitochondrial proton leak), this phenomenon will be discussed in detail in future sections. The physiological states that are associated with a decreased metabolic efficiency (or increased inefficiency) include exposure to cold, excessive caloric intake and being newborn. In contrast to exercise-induced and cold-induced shivering thermogenesis (processes which lead to ATP synthesis and breakdown in muscle, and do not represent a true state of metabolic inefficiency), cold-induced non-shivering thermogenesis and DIT are metabolically inefficient processes (heat production without synthesis of ATP). Cold-induced non-shivering thermogenesis and DIT both take place in BAT via UCP1-mediated mitochondrial uncoupling, and are referred to as processes of adaptive thermogenesis, a subcategory of facultative energy expenditure (Himms-Hagen, 1990; Nicholls and Locke, 1984). The two major regulators of adaptive thermogenesis are β-adrenergic agonists and thyroid hormones. They exert their effect on energy expenditure by altering the amount of mitochondria in skeletal muscle and BAT, and the expression of genes that are controllers of mitochondrial fuel-oxidation and ATP synthesis (Nicholls *et al.*, 1986).

1.3. Oxidative Phosphorylation and the Mitochondrial Proton Leak

Oxidative phosphorylation is the overall process whereby adenosine triphosphate (ATP) is synthesized from adenosine diphosphate (ADP) and inorganic phosphate (P_i) by mitochondria (Figure 1). It is driven by electron flow along a series of mitochondrial enzymes from a reduced substrate to oxygen. Electron transport results in a concomitant translocation (pumping) of protons at three energy-transducing complexes in the respiratory chain (Complex I, III and IV) from the mitochondrial matrix into the intermembrane space. This forms an electrochemical potential across the mitochondrial inner membrane, known as the protonmotive force (Δp). Protonmotive force is composed of a pH gradient (ΔpH) and an electric membrane potential ($\Delta\Psi_m$). Δp allows ATP synthesis by driving protons back into the matrix through F_oF_1-ATPase; the combined action of ATP synthase, adenine nucleotide translocator and the phosphate carrier results in the formation of ATP.

It has long been recognized that the coupling of oxidative phosphorylation is not perfect and that the F_oF_1-ATPase is not the only route by which protons can return into the mitochondrial matrix (Mitchell and Moyle, 1967; Nicholls, 1974). Research into this phenomenon has conclusively demonstrated that mitochondrial membranes passively leak protons such that a significant proportion of protons return to the mitochondrial matrix without their entry being coupled to ATP synthesis or any other energy conserving reaction (Brown, 1992; Brown and Brand, 1986) (Figure 1). Flux of protons through this nonproductive proton conductance pathway has been termed 'proton leak'. Another phenomenon that may result in the uncoupling of oxidative phosphorylation is known as

FIGURE 1. SCHEMATIC ILLUSTRATION OF THE MITOCHONDRIAL INNER MEMBRANE SHOWING MAJOR COMPONENTS OF THE OXIDATIVE PHOSPHORYLATION PATHWAY AND UNCOUPLING PROTEIN-MEDIATED MITOCHONDRIAL PROTON LEAK. Shown in turquoise are the different Complexes of the electron transport chain. As reducing electrons are passed from one Complex to another, protons are being pumped from the mitochondrial matrix into the intermembrane space with the establishment of an electrochemical gradient, which serves as a reservoir of free energy. This energy, also referred to as protonmotive force, can be trapped as protons are channelled through the ATP synthase (pink) and the combined action of the adenine nucleotide (purple) and the phosphate carriers (grey), resulting in the creation of ATP. The uncoupling protein-mediated proton leak (green) is a non-productive mode of proton translocation in which protons passively leak back into the matrix without being coupled to ATP formation or any other energy conserving reaction. The protonmotive force is dissipated and this energy is simple released as heat. The oxygen consumption occurring during cellular respiration therefore maintains both ATP turnover and proton leak reactions in varying proportions depending on cellular energy demands.


Mitochondrial intermembrane space
LEAK
ATP
ADP
H+
H+
H2PO4-
H+
Complex II
Ubiquinone
Cyt c
Complex IV
Complex I
Complex III
H+
H+
H+
H2PO4-
ADP
H+
ATP
Reducing equivalents
e- + O2 + H+ → H2O
Mitochondrial matrix

redox slip. Redox slip is electron flow without proton pumping to the intermembrane space (Pietrobon *et al.*, 1981). However, Brand and colleagues (Brand *et al.*, 1994b) have shown that at physiologic temperatures, slip reactions are insignificant and need not be accounted for when protonmotive force is high.

The first indications of leak were made by Mitchell and Moyle (Mitchell and Moyle, 1967) through oxygen pulse experiments. They achieved this by adding a small pulse of oxygen to anaerobic mitochondria or bacteria, which then pumped out a pulse of protons detected by a pH electrode. When the oxygen was used up, the protons leaked back across the membrane. This proton conductance was initially believed to be an artifact of damage to mitochondria, sustained in the act of their isolation. An important first step in determining if proton leak was indeed an experimental artifact was to measure proton leak rate at a high Δp by first inhibiting all known proton transport processes and then inducing an artificial Δp with potassium gradients (Brown and Brand, 1986; Krishnamoorthy and Hinkle, 1984). The rate of leak was shown to be much greater at high Δp values and had an exponential dependence on Δp (*i.e.*, leak is non-ohmic). The strong dependence on Δp explains the finding of Nicholls which showed that when mitochondria are respiring in the absence of ATP synthesis (state 4 respiration), respiration can be substantially reduced by titrating with respiratory chain inhibitors with only a small decrease in Δp. Further proof that the proton leak is not an experimental artifact came from studies in which mitochondria were subjected to experimental protocols which did not involve mechanical or chemical disruption of the cell. This was achieved by measuring mitochondrial membrane potential at different rates of cellular oxygen consumption in isolated rat hepatocytes (Nobes *et al.*, 1990a). These

experiments demonstrated that proton leak across the inner membrane was as great when mitochondria were functioning in intact cells as it was in isolated mitochondrial preparations. This has been confirmed in further studies in rat thymocytes (Buttergeit *et al.*, 1992), pig spleen lymphocytes (Buttergeit *et al.*, 1991), brain (Rolfe *et al.*, 1994), kidney (Rolfe *et al.*, 1994), and perfused skeletal muscle (Rolfe and Brand, 1996b). Thus, basal proton conductance of the mitochondrial inner membrane is a feature of undamaged mitochondria operating in their natural environment, the cytosol.

1.4 Physiological Significance of the Proton Leak

The quantitative importance of proton leak in energy expenditure is substantial. Recent findings suggest that the uncoupling that is mediated by the mitochondrial proton leak accounts for 20-25% of the standard metabolic rate (SMR), making it the single-most important predictor of SMR (Rolfe and Brand, 1996a). Studies with intact cells have allowed the measurement of cellular metabolic rates (*i.e.*, oxygen consumption) required to sustain the proton leak and allowed the comparison of this cost with the overall rate of oxygen consumption. It has been shown that the energy consumed by the leak represents about 22-25% of total cellular oxygen consumption rate or 35-45% of mitochondrial respiration rate in rat hepatocytes (Brand, 1990b; Brand *et al.*, 1994a; Brown *et al.*, 1990b; Harper and Brand, 1993; Nobes *et al.*, 1990a). Although the liver consumes a significant proportion of total cellular oxygen consumption, it only accounts for 10-20% of SMR of a rat (Field *et al.*, 1939) and represents a small proportion of total tissue mass. Skeletal muscle, on the other hand, occupies the largest proportion of body mass, making it the single

most important contributor to rat SMR (accounting for 33-40% of SMR (Field *et al.*, 1939)) (Rolfe and Brand, 1996a). Thus, a more useful estimate of the significance of the proton leak has come from measurements in muscle. Studies by Rolfe and Brand (Rolfe and Brand, 1996a) have demonstrated that up to 52% of metabolic rate in perfused resting (*i.e.*, not contracting) rat hindquarter muscles is dedicated to countering the leakage of protons into the mitochondrial matrix. It is thus based on this high proportion of oxygen consumption used to drive the proton leak, that it has been hypothesized that the latter may have a significant influence over mammalian energy expenditure and SMR.

1.5. Postulated Functions of the Mitochondrial Proton Leak

Several different functions have been suggested for the proton leak in mammals. These functions include: i) thermogenesis, ii) increased capability for regulation of energy metabolism, iii) the mitigation of the production of harmful free radicals.

1.5.1. Proton Leak and Thermogenesis

It has often been proposed that the mitochondrial proton leak in mammals represents the cost of endothermy, and thus the matching of heat production and heat loss. The bulk of heat produced in the mammal is mainly by metabolically active organs such as the liver, kidney, heart and muscle. Relative to body size, the mass of these organs is relatively larger in small animals. Larger animals have a smaller surface area to volume ratio, which has been correlated to a decrease in mass-specific metabolic rate (the metabolic rate per unit body mass) (Brand, 1990a). In contrast, smaller animals tend to lose a large amount of heat since

they have a larger ratio of surface area to volume (Brand, 1990a). This loss in heat is balanced by an increase in metabolic rate (Brand, 1990a). Krebs (Krebs, 1950) found that metabolic rate in individual organs was greater for small animals, for example, mouse liver slices consumed oxygen four times faster than horse liver slices. In 1985 Else and Hulbert (Else and Hulbert, 1985) demonstrated that changes in the rate of oxygen consumption per unit mass of that tissue are paralleled by changes in the content of mitochondria and mitochondrial components. They found that in small mammals the mitochondria of liver and brain had more surface area/volume of mitochondria and mitochondria of liver, kidney and heart occupied a greater proportion of the cell. It was not until 1993 that Porter and Brand (Porter and Brand, 1993) discovered that the rate of mitochondrial proton leak is inversely related to body mass, as is metabolic rate. Their finding that liver mitochondria from mice are about 4-fold more leaky to protons than liver mitochondria from horses provided an explanation for Kreb's initial finding (Krebs, 1950) that metabolic rate is increased in the tissues of small animals. Poikilotherms (invertebrates, fish, amphibia and reptiles) have a 4-5 fold lower oxygen consumption rate than a homeothermic mammal of the same body mass. Brand and colleagues (Brand *et al.*, 1991) found that isolated liver mitochondria of the bearded dragon lizard (*Amphibolurus vitticeps*) has a lower mitochondrial proton leak activity than a rat of equivalent body size and preferred body temperature (Brand *et al.*, 1991). This provides further evidence indicating that proton leak plays an important role in overall energy expenditure. However, the similarity of the proportion of energy consumed by the leak and other oxygen consuming processes in rat and lizard liver mitochondria implies that heat production is not the solitary function of the leak.

1.5.2. Proton Leak Increases Potential for Regulation of Oxidative Metabolism

Because the oxygen consumption that is used to support the seemingly futile proton leak across the mitochondrial membrane cannot be used to drive ATP synthesis, the occurrence of leak in intact cells lowers the effective P/O ratio (actual ATP produced/O_2 consumed) below its maximal possible vaulues (Brand, 1994). It follows that the contribution of the leak to total proton influx declines as ATP synthesis increases since a greater proportion of protons return through ATP synthase (Brown *et al.*, 1990a). In the latter case, the control by leak over respiration declines correspondingly. Hence, the presence of the proton leak allows oxidative phosphorylation to vary its coupling efficiency depending on ATP demand without large changes in oxygen. Proton leak may also increase sensitivity and decrease response time to changes in ATP utilization in the cell. For example, when ATP demand is high, the proton leak and coincident oxygen consumption could be minimized in order to fulfill ATP requirements.

1.5.3. Proton Leak as a Means of Reducing Free Radical Production

The rate of generation of superoxide free radicals from oxygen is directly related to oxygen concentration within the cell (Halliwell and Gutteridge, 1989) and the degree of reduction of electron donors. In the resting state, energy consumption is low and the availability of ADP for phosphorylating respiration decreases, which may yield higher intracellular levels of oxygen and of reduced electron transport chain components (*e.g.*, NADH) which could donate an electron to oxygen to form superoxide anion, hydrogen

peroxide and hydroxyl radical, and then cause oxidative damage to the cell. It has been proposed by Skulachev (Skulachev, 1996) that the uncoupling of mitochondrial respiration allows the maintenance of low levels of both oxygen and reactive oxygen species (ROS) when phosphorylating respiration fails to do so due to a lack of ADP. In other words, an increase in proton leak would thus stimulate oxygen consumption and decrease the formation of harmful oxygen radicals (Skulachev, 1996).

1.6. Mechanisms of Proton Conductance

Despite many years of study, the mechanism(s) by which proton leak/conductance occurs remains unknown. Much debate has centred around whether the leak is protein-mediated, or merely a diffusion of protons through the phospholipid bilayer of the mitochondrial inner membrane. The latter mechanism was tested when experiments with mitochondrial inner membrane phospholipids reconstituted into liposomes showed that the phospholipid fatty acid composition of the inner membrane is strongly correlated with proton conductance of the membrane (Brookes *et al.*, 1997b; Porter *et al.*, 1996). However, aspects other that phospholipid composition influence proton permeability since proton leak in protein-free membranes only supported 5% to about 25% of total leak in liver mitochondria of rats and lizards, respectively (Brookes *et al.*, 1998; Brookes *et al.*, 1997a). These results are consistent with at least two hypotheses: (1) the physical properties of the artificial membrane (*e.g.*, packing effects, surface charge, radius of curvature) are altered by the removal of the proteins; or (2) the proteins (or protein) which facilitate some or most of the proton leak have been removed. In the late 1970s Nicholls and colleagues demonstrated

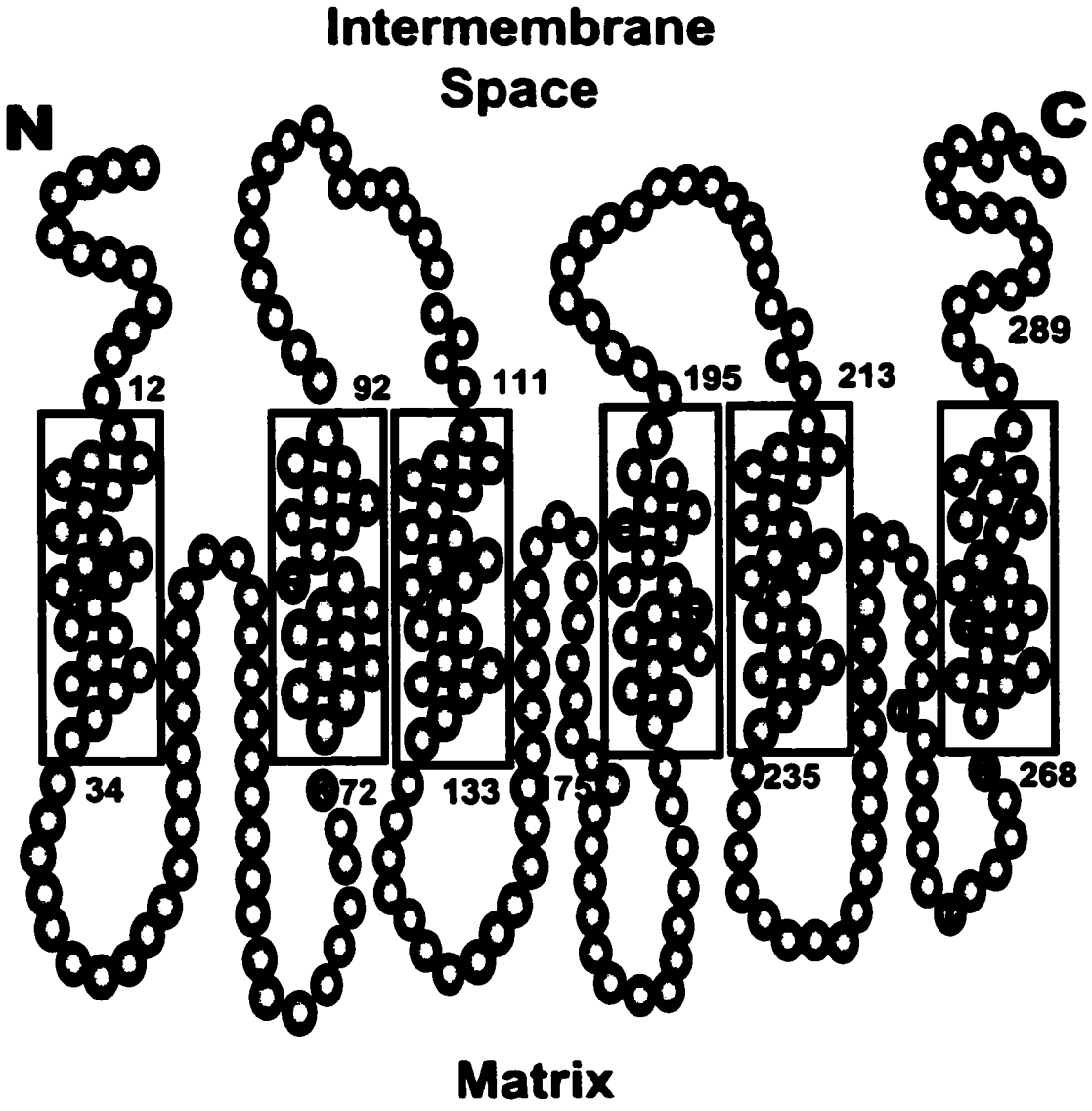
that there indeed does exist a protein-mediated mechanism by which oxidative phosphorylation can be uncoupled (Nicholls, 1977; Nicholls *et al.*, 1978). It was revealed that a 32 kDa protein, referred to as uncoupling protein-1 (UCP1) or thermogenin resides in the mitochondrial inner membrane of mature brown adipose tissue mitochondria and plays a role in generating heat by creating a pathway that allows dissipation of protonmotive force across the mitochondrial inner membrane (Nicholls and Locke, 1984).

1.7. The Uncoupling Protein (UCP) and Its Homologues

1.7.1. UCP1 - Structural Aspects

In 1985, the cDNA for Ucp1 was cloned by Bouillaud and colleagues (Bouillaud *et al.*, 1985). The early cloning of Ucp1 was made possible because of its high expression in BAT, and the inducible character of its mRNA. The cDNA indicated the complete sequence of Ucp1, and direct amino acid sequencing unravelled its primary structure (Aquila *et al.*, 1985). Analyses of its amino acid sequence have shown that UCP1 has homology to members of the mitochondrial carrier protein family such as the ATP/ADP translocator and the phosphate carrier (Aquila *et al.*, 1985; Aquila *et al.*, 1987). These similarities include six transmembrane helices and the location/appearance of both the C- and N-termini protruding into the mitochondrial intermembrane space, as shown in Figure 2 (Klingenberg, 1999; Klingenberg and Huang, 1999). The sequence is divided into three homologous internal domains: three repeats containing about 100 amino acid residues each with two transmembrane helices. The two helices are separated by an average of 40-residue long, highly hydrophilic sequences located at the matrix side of the mitochondrial inner membrane.

FIGURE 2. MEMBRANE-SPANNING MODEL OF THE UNCOUPLING PROTEIN-1 (UCP1). UCP1 is proposed to contain six transmembrane helices spanning the mitochondrial inner membrane, with both the C- and N-termini protruding into the mitochondrial intermembrane space. The structure can be divided into three similar repeat domains of about 100 residues, each containing two transmembrane helices. Within each domain, two helices are separated on the matrix side by a 40 (approx.) residue long hydrophilic stretch of amino acids.


Intermembrane
Space
N
C
12
92
111
195
213
289
34
72
133
175
235
268
Matrix

UCP1 has some physiologically significant, and experimentally useful, properites: it can be 'turned on' by fatty acids and 'turned off' by purine di- and tri-phosphates. The regulation of UCP1 activity will be further discussed below.

1.7.2. UCP1-MEDIATED PROTON CONDUCTANCE IN BROWN ADIPOSE TISSUE

The best-studied example of protein-mediated proton conductance is found in studies with mitochondria from BAT. The physiological function of BAT, and of UCP1, is thermogenesis (Nicholls and Locke, 1984). Thermogenesis, and thus energy expenditure in this unique tissue is stimulated by the sympathetic nervous system and hence dramatically increases in oxygen consumption. Norepinephrine released from nerve terminals stimulates β-adrenergic receptors on the plasma membranes of brown adipocytes, which results in uncoupled respiration. Small mammals adapt to cold by increasing the amount of UCP1 their BAT mitochondria, which (under β-adrenergic stimulation) become more permeable to protons (Cannon *et al.*, 1999). This results in an augmented rate of proton conductance and increased heat production. The heat produced is subsequently dissipated into the circulation by arteriovenous anastomoses in BAT, thus allowing small mammals to achieve homeothermy in the face of temperatures below normal (non-shivering thermogenesis) or after ingestion of excessive amounts of calories (diet-induced thermogenesis). That a transgenic mouse with ablated BAT is obese (Lowell *et al.*, 1993), and that this phenotype disappears at thermoneutrality, strongly supports evidence that BAT, and UCP1, play an important roles in the regulation of energy balance in rodents.

1.7.3. UCP2 - The Second Homologue

In 1997, discoveries in the field of molecular biology opened the door for further studies into the mechanism of proton leak. Up until this time, UCP1 was the only known protein uncoupler of mitochondrial respiration. However, this protein is selectively expressed in BAT (and has recently been identified in uterine smooth muscle (Nibbelink *et al.*, 2001)) and therefore could not be involved in proton conductance in the mitochondria of other metabolically active organs such as liver and muscle. The question of great interest at this time was: Is a protein responsible for mediating proton conductance in non-BAT mitochondria? Fleury *et al* (Fleury *et al.*, 1997) and Gimeno *et al* (Gimeno *et al.*, 1997) independently discovered and described a protein with a notably high (59%) amino acid identity to UCP1, and named it UCP2. Several protein motifs known to be important in UCP1 function are also conserved in UCP2, including the putative nucleotide binding sequences (see Section 1.10.1.). The amino acid sequence of mouse UCP2 (mUCP2) is 95% identical to human UCP2 (hUCP2) (Fleury *et al.*, 1997). An important feature of UCP2 is that it is ubiquitously expressed in nearly all cell types and could thus be a candidate for the mechanism of basal proton leak. Ucp2 was also shown to be upregulated in white adipose tissue (WAT) in response to a high-fat diet (Fleury *et al.*, 1997), and thus could be responsible in part for diet-induced thermogenesis (DIT). In contrast to Ucp1, levels of mouse Ucp2 mRNA remained unchanged in BAT and WAT in the presence of the β_3-adrenergic agonist CL316,243, or following cold exposure (Fleury *et al.*, 1997). UCP2 was also shown to be able to uncouple mitochondrial respiration, as assessed by flow cytometry, when transfected using an inducible expression vector into *Saccharomyces cerevisiae* cells

(Fleury *et al.*, 1997; Gimeno *et al.*, 1997). When protein expression was induced, a significant drop in mitochondrial membrane potential was observed. In addition, mitochondrial respiration measurements in isolated yeast mitochondria also showed lowered coupling in UCP2-containing mitochondria (Fleury *et al.*, 1997). These observations, and the striking similarity of UCP2 to UCP1, have been taken as evidence for the protein's uncoupling function, but not without debate, as will be discussed below.

1.7.4. UCP3 - One More UCP Homologue

Subsequent to the discovery of Ucp2, another mammalian Ucp was discovered, namely, Ucp3. At the amino acid level, hUCP3 has 73% identity to hUCP2 and 57% identity to hUCP1 (Boss *et al.*, 1997b; Vidal-Puig *et al.*, 1997). UCP3, like UCP1, has a much more limited tissue distribution. UCP3 is distinguished from UCP2 by its relatively selective and abundant expression in skeletal muscle in humans, and in BAT and skeletal muscle in rodents, making it a potential mediator of adaptative thermogenesis (Matsuda *et al.*, 1997; Vidal-Puig *et al.*, 1997).

1.7.5. UCP4 and BMCP-1 - The Final Homologues?

The most recent additions to the UCP family are UCP4 (Mao *et al.*, 1999) and brain mitochondrial carrier protein-1 (BMCP1) (Sanchis *et al.*, 1999). UCP4 has greatest homology to UCP3, however its transcripts are exclusively found in fetal and adult brain tissues. With only 29% similarity to UCP1, UCP4 is no more similar to UCP1 than the latter is to the mitochondrial carriers (Runswick *et al.*, 1987). Northern analysis of mouse, rat,

and human tissues demonstrated that mRNA of the BMCP1 gene is mostly expressed in the brain, although it is has also been identified in low quantities in other tissues. The sequence similarity of 34, 38, or 39% between BMCP1 and Ucp1, Ucp2, or Ucp3, respectively, is also low. Ectopic expression of Ucp4 genes has been shown to reduce mitochondrial membrane potential in mammalian cells (Mao *et al.*, 1999), as does the expression of BMCP1 genes in yeast spheroblasts (Sanchis *et al.*, 1999).

1.8. Chromosomal Location of UCP1 and Its Homologues

The Ucp1 gene is located on chromosomes 8 and 12 in mice and humans, respectively (Cassard *et al.*, 1990). The chromosomal localisation of the Ucp2-Ucp3 cluster is 11q13 between D11S916 and D11S911 in humans and chromosome 7 in mice, a location which is in the vicinity of markers linked to hyperinsulinemia and obesity (DeBry andSeldin, 1996; Kleyn *et al.*, 1996; Taylor andPhillips, 1996). These two genes are tightly linked, separated by approximately 7.9 kb of genomic DNA in humans and by 8.3 kb in mice (Fleury *et al.*, 1997; Solanes *et al.*, 1997), which means that they could have emerged from a gene duplication event (Solanes *et al.*, 1997). The intron/exon structure of the three genes are similar in that each of the six transmembrane domains is encoded by an exon. The only difference is that the exon structure differs at the 5′ end of each gene. *Ucp1* has six exons in total, with the first exon encoding 231 bases of the 5′ untranslated region (UTR) and the first 41 amino acids. In contrast, Ucp2 has eight exons, with the two additional ones being used to encode the 5′ UTR. Ucp3 has seven exons, with the 5′ UTR encoded by two exons. BMCP1 is located on the X chromosome (Xq25-26) in humans (Bouillaud *et al.*, 2000),

between genetic markers DXS1206 and DXS1047. Ucp4 has been mapped to chromosome 6p11.2-q12 close to the genetic marker SHGC-34952 (Mao *et al.*, 1999).

1.9 Functional Studies of the UCP1 Homologues and Proton Conductance

To date, there has been no concrete evidence proving beyond a reasonable doubt that the UCP1 homologues are indeed proton-conducting uncouplers of respiration, or have a definite thermogenic function. The fundamental question remains: could any of the members of the UCP family be responsible for the observed basal level of uncoupling in all mitochondria? Several observations suggest that they are not. UCP1 homologues are not found in all organisms (but have been found in all mammals examined to date), nor are they in all cell types within an organism (*e.g.*, UCP2 found in Kupfer cells of the liver, but not hepatocytes (Carretero *et al.*, 1998)), therefore they cannot account for the observed proton conductance that is known to occur in all eukaryotic mitochondria (Stuart *et al.*, 1999). To test the hypothesis that UCP1 homologues cause leak, many researchers have genetically manipulated the expression of the various uncoupling proteins and measured the effect on mitochondrial function. These studies are discussed below.

1.9.1 Heterologous Expression in Recombinant Yeast

Several groups have successfully expressed the UCP proteins in yeast and their results appear to confirm their uncoupling activities (*i.e.*, lowering of membrane potential and increase in state 4 respiration) (Boss *et al.*, 1997b; Fleury *et al.*, 1997; Gimeno *et al.*, 1997; Gong *et al.*, 1997; Rial *et al.*, 1999; Sanchis *et al.*, 1999; Vidal-Puig *et al.*, 1997; Zhang *et al.*, 1999). However, studying the function of UCPs in this way has not gone

without criticism. Stuart *et al* (Stuart *et al.*, 2001; Stuart *et al.*, 2000) have put forth three major criticisms of studies where UCPs have been expressed in yeast, they are as follows: (1) Ucp3 expression inhibits FCCP-uncoupled rates of respiration. Physiologically, there is no reason, related to the uncoupling activity of a UCP, for this to happen. This may indicate that mitochondrial substrate oxidation may be disturbed by the expression of a heterologous protein; (2) the presence and function of the UCP1 homologues are sometimes inferred from the appearance of an uncoupling phenotype. In other words, expression conditions may be altered until an uncoupling phenotype is observed (*i.e.*, until enough protein has been expressed and it is 'active'), such an approach favours the exclusion of negative findings; (3) we do not know how the amount of protein expressed in yeast relates to the amount of protein that may be present in mammalian mitochondria. The amount of protein can potentially be much greater in yeast mitochondria, and this alone could readily account for incongruent results with mitochondria from mammalian tissues. (Stuart *et al.*, 2000).

1.9.2 Proteoliposome Reconstitution Studies

An alternative approach to studying the regulation and function of UCP1 homologues has been to reconstitute these proteins into liposomes after protein expression in *E. coli* (Echtay *et al.*, 1999; Jaburek *et al.*, 1999). One obstacle in using this method is that proteins expressed in *E. coli* accumulate in large quantities in inclusion bodies. Therefore, following isolation and solubilisation, the proteins must be correctly renatured in order to recover their native function before they can reconstituted into the liposomes

(Echtay *et al.*, 1999). Since the native function of the homologues is still not known, UCP1 is usually reconstituted under identical conditions, thus acting as an experimental control.

A recent study published by Echtay and colleagues (Echtay *et al.*, 1999) demonstrates that it is possible to recover a purine nucleotide-sensitive chloride anion (Cl^-) transport in UCP1 reconstituted from *E.coli*. Proton transport, however, was not recovered, but was reinstated using the same methods for UCP1 reconstituted from hamster BAT mitochondria. No proton transport was indicated for hUCP3 reconstituted bacteria (with or without the fatty acid, laurate), but chloride transport by UCP3 was indeed inhibited by purine nucleotides. The authors could not explain the defect in proton transport. They suggest that since chloride anions and protons are both controlled from the same nucleotide binding site, the inhibition of Cl^- transport by nucleotides should be representative for the regulation of any potential proton transport activity of UCP in mitochondria. Around the same time that Echtay's findings were published, Jaburek *et al.* also published their findings from bacterially expressed UCP2 and UCP3 (Jaburek *et al.*, 1999). Their ion-flux studies demonstrated that bacterially expressed UCP2 and UCP3 in liposomes catalysed a fatty-acid dependent electrophoretic proton flux. Proton conductance could be inhibited by purine nucleotides but with much lower affinity than observed with UCP1 (K_i values 3000 times greater than those determined for inhibition of UCP3 chloride transport by Echtay (Echtay *et al.*, 1999)). However, the authors did not use bacterially expressed UCP1 as a control to demonstrate that uncoupling activity could be restored under conditions identical for UCP2 and UCP3, or that its inhibition by nucleotides is similar to that in BAT mitochondria.

The weakness of these types of studies is that they are limited by the fact that

inclusion body preparations can by contaminated with bacterial proteins that may introduce artifactual activities into the liposomal preparation, and the artificial lipids used to reconstitute the proteins are not representative of the lipid composition of the mitochondrial inner membrane. In the two studies mentioned above, it is quite possible that the proteins could have been incorrectly refolded. This could explain the low nucleotide affinities of UCP2 and UCP3 in Jaburek's study (Jaburek *et al.*, 1999), and the lack of UCP3 proton transport in Echtay's study (Echtay *et al.*, 1999). But this hypothesis is only plausible if UCP2 and UCP3 normally exhibit high nucleotide affinity, and UCP3 is a confirmed proton translocator. This cannot be decided until the true function of these proteins is known. Taken together, reconstitution experiments do not, as of yet, conclusively demonstrate that UCP2 and UCP3 are proton translocating uncouplers *in vivo*.

1.9.3 Transgenic Overexpression and Gene Knockout Studies

The application of transgenic technologies has allowed the development of transgenic mice as an approach to investigate the physiological function of UCP1 and its homologues. Recombinant DNA technology is used as a tool to remove a gene (or part of a gene), or to overexpress a gene of choice . The resulting mice are commonly referred to as either 'knockout' or 'overexpression' mice, respectively, for a particular gene.

Although gene knockout and overexpression approaches are useful tools, there are difficulties associated with the use of these methods. One of the problems associated with the use of knockout mice is that another gene which encodes a protein with homologous function to the knocked out gene may be indirectly upregulated. This may lead to partial or

complete compensation of the physiological function of the targeted genes. Thus, the conclusions from studies with knockout mice may not reflect the true function of the targeted gene. Another common problem with the use of knockout mice is that the removal of a gene may interrupt normal embryonic development, or render the transgenic animal infertile. The use of transgenic mice overexpressing a certain gene also does not go without problems. The transfer of a foreign transgene construct cannot be guaranteed to be properly integrated into the genome. Therefore, multiple copies of the transgene may be inserted, and multiple copies of the gene made. Taking into account all of the drawbacks associated with knocking out and overexpressing genes, transgenic approaches are still one of the most useful and powerful tools that can be used to elucidate the true importance and function of a specific protein under physiological conditions (See Harper and Himms-Hagen, 2001 for a review).

1.9.3.1 THE UCP1 KNOCKOUT MOUSE

The first Ucp knockout mouse created was the *Ucp1*-knockout mouse. The importance of BAT metabolism in the development of obesity in experimental animals, and the obese phenotype observed when BAT is ablated (Lowell *et al.*, 1993), led a group directed by Leslie Kozak to create a transgenic mouse model in which Ucp1 gene was inactivated (Enerbäck *et al.*, 1997). It was expected that mice having gene-targeted inactivation of Ucp1 would become obese and diabetic at a young age. It was obviously surprising when *Ucp1*-knockout mice showed no enhanced susceptibility to obesity, even when fed a high fat diet (58 kcal% as fat). *Ucp1*-knockout mice are, however, characterized

by a cold-sensitive phenotype, providing evidence that UCP1 plays an important role in thermogenesis. For my Master's thesis [Monemdjou, 1999 #2586], I was fortunate enough to be afforded access to these unique mice in order to examine the bioenergetics of isolated mitochondria lacking UCP1. We were able to show that GDP inhibits proton leak in mitochondria from control mice by approximately 50%, however, under the same incubation conditions GDP has no effect on leak in mitochondria isolated from *Ucp1*-knockout mice (Monemdjou, 1998; Monemdjou *et al.*, 1999). Experiments were performed in the presence of 0.5% defatted bovine serum albumin (BSA) in order to study the mitochondria in a fatty acid free environment. Because BAT expresses Ucp2 and Ucp3 (in rodents), these results show that any proton leak that is caused by either of these UCPs is insensitive to GDP (although they both do possess putative nucleotide binding domains). It was also interesting that despite a 5-fold increase in Ucp2 mRNA in the absence of Ucp1 (Enerbäck *et al.*, 1997), we did not observe any differences between controls and *Ucp1*-knockout mice in the amount of proton leak in the presence of saturating amounts of GDP (Monemdjou, 1998; Monemdjou *et al.*, 1999). Thus, the relative levels of Ucp2 mRNA do not correspond to any differences in uncoupling when studied under our experimental conditions.

Since BAT plays a minor role in energy balance in adult humans, we decided to examine the characteristics of the proton leak in skeletal muscle mitochondria of *Ucp1*-knockout mice. The finding of major interest from this study was that the overall kinetics of the mitochondrial proton leak showed that oxygen consumption used to support the eak is higher in mitochondria of *Ucp1*-knockout mice compared to controls (Monemdjou, 1998; Monemdjou *et al.*, 2000). These results supported our hypothesis that skeletal muscle may

be a potential site for an adaptative thermogenic mechanism favouring the lean phenotype observed in the *Ucp1*-knockout mouse.

1.9.3.2 THE UCP3-KNOCKOUT MOUSE

Two groups have recently reported on the creation of a transgenic mouse lacking the gene for Ucp3 (*Ucp3*-knockout mice). The first group, headed by Marc Reitman, produced the *Ucp3*-knockouts by using a 129/SvC embryonic stem cell line to produce chimeric mice which were then crossed to Swiss Black mice (Gong *et al.*, 2000). Under the supervision of Brad Lowell, a second group produced the knockouts by electroporating the targeting vector into J1 embryonic stem cells and the targeted clones were then injected into C57BL6 embryos (Vidal-Puig *et al.*, 2000). Both groups demonstrated that *Ucp3*-knockout mice were neither obese or cold-sensitive, nor was there an increase in other Ucp mRNAs. Despite lacking UCP3, the knockout mice had normal serum insulin, triglyceride, and leptin levels, with a tendency toward reduction in free fatty acids and glucose (Gong *et al.*, 2000). Mice lacking UCP3 displayed normal circadian rhythms in body temperature and motor activity and had normal body temperature responses to fasting, stress, thyroid hormone, and cold exposure (Gong *et al.*, 2000). Vidal-Puig and coworkers showed that mitochondria from *Ucp3*-knockout mice are more coupled, as measured by an increase in respiratory control ratio, and had increased production of reactive oxygen species (ROS) (Vidal-Puig *et al.*, 2000). This could be evidence supporting a role for UCP3 in preventing oxidative damage in skeletal muscle.

Our collaboration with Dr. Reitman allowed us the opportunity to study the

metabolic characteristics of the *Ucp3*-knockout mouse, and have our findings published in the original paper (Gong *et al.*, 2000). Based on the high homology of UCP3 with UCP1, and on its high levels of expression in skeletal muscle and BAT, we hypothesized that UCP3 could potentially play an important role in energy balance and thermogenesis. Unlike UCP1, UCP3 is highly expressed in adult humans, and thus could be a potential target site for drug therapy for the treatment of obesity. Our results from human clinical studies will be discussed later in this thesis.

1.9.3.3 THE UCP1-UCP3 DOUBLE KNOCKOUT

The *Ucp1-Ucp3* double knockout mouse was created in order to determine whether the phenotype of the *Ucp1*-knockout mouse would be made more severe by the genetic removal of Ucp3 (Gong *et al.*, 2000). The double knockout mice did not demonstrate any further reduction in their response to β-adrenergic stimulation, and were not more sensitive to cold compared to mice deficient in UCP1. This proves that Ucp3 is not a modifier gene of the *Ucp1*-knockout phenotype.

1.9.3.4 MICE OVEREXPRESSING HUMAN UCP3

The physiological effects of Ucp overexpression was finally examined very recently. With much success, Clapham and colleagues at SmithKline-Beecham created mice that overexpress hUCP3 in skeletal muscle of C57BL6 x CBA mice, and named them UCP-3Tg mice (Clapham *et al.*, 2000). Total Ucp3 mRNA expression was increased 66-fold in skeletal muscle. Other than in BAT, where there was a slight change in Ucp3 mRNA

expression, there was little or no expression in other tissues. By 8 weeks of age, UCP-3Tg males had a significantly lower body weights (even when fed a high-fat diet), despite increased food intake, compared to wild-type mice. Mice overexpressing Ucp3 were also more glucose-tolerant than wild-type mice. Based on these interesting findings, the idea that UCP3 does indeed play a role in metabolic efficiency and whole body energetics was once again put forth as a valid hypothesis. Our collaboration with Dr. Clapham enabled us to have access to these mice in order to conduct detailed analyses on the metabolic characteristics, specifically proton leak, of skeletal muscle mitochondria of these special mice. Our findings from this study will be discussed in this thesis.

1.9.4 Effect of Physiological Perturbations on UCP Expression

Results from experiments on animal models that have been physiologically perturbed do not support an uncoupling function for the UCP1 homologues. In rats, levels of Ucp2 and Ucp3 mRNA in muscle rose in response to starvation (Samec *et al.*, 1998b), despite the fact that under these conditions thermogenesis in muscle is known to be depressed. Despite the observations that Ucp2 and Ucp3 mRNA levels were increased more than 5-fold and 4-fold, respectively, and UCP3 protein levels rising 2-fold, no changes in proton conductance was detected (Cadenas *et al.*, 1999). One study with mice has shown that starvation induces a 3.5-fold increase in soleus muscle Ucp3 mRNA expression, but there was no accompanying soleus muscle heat production (Boss *et al.*, 1998c). These paradoxical findings will be elaborated on in the Discussion section.

1.10. Regulation of Mitochondrial Proton Leak by UCP1 and its Homologues

1.10.1 Purine Nucleotide Inhibition of Proton UCP1-mediated Leak

It has been over two decades that purine nucleotides were shown to bind and inhibit proton conductance by UCP1 (Desautels *et al.*, 1978; Heaton *et al.*, 1978; Lin and Klingenberg, 1982). The inhibition of proton transport by purine nucleoside di- and tri-phosphates (*i.e.*, ADP, ATP, GDP and GTP) was key in tracking down the source of uncoupling in BAT. In studies with mitochondria, GDP and GTP were found to be preferred over ADP and ATP (Klingenberg and Huang, 1999). Also, binding and inhibition are inversely related to pH (Klingenberg and Huang, 1999). Site-directed mutagenesis studies have revealed that three arginine residues that are located on helices 2, 4, and 6, are essential for complete purine nucleotide binding to, and the inhibition of, UCP1-mediated leak (Modriansky *et al.*, 1997). Both UCP2 and UCP3 possess all of the residues thought to be essential for purine nucleotide binding in UCP1 (Boss *et al.*, 1997b; Fleury *et al.*, 1997; Gimeno *et al.*, 1997; Vidal-Puig *et al.*, 1997), however, recent studies have shown that a considerably higher concentration of nucleotide is needed to inhibit these proteins (Jaburek *et al.*, 1999). In UCP2, as in UCP1, ATP is a stronger inhibitor of proton conductance than ADP, but in UCP3, ADP inhibits more strongly than ATP (Echtay *et al.*, 2001).

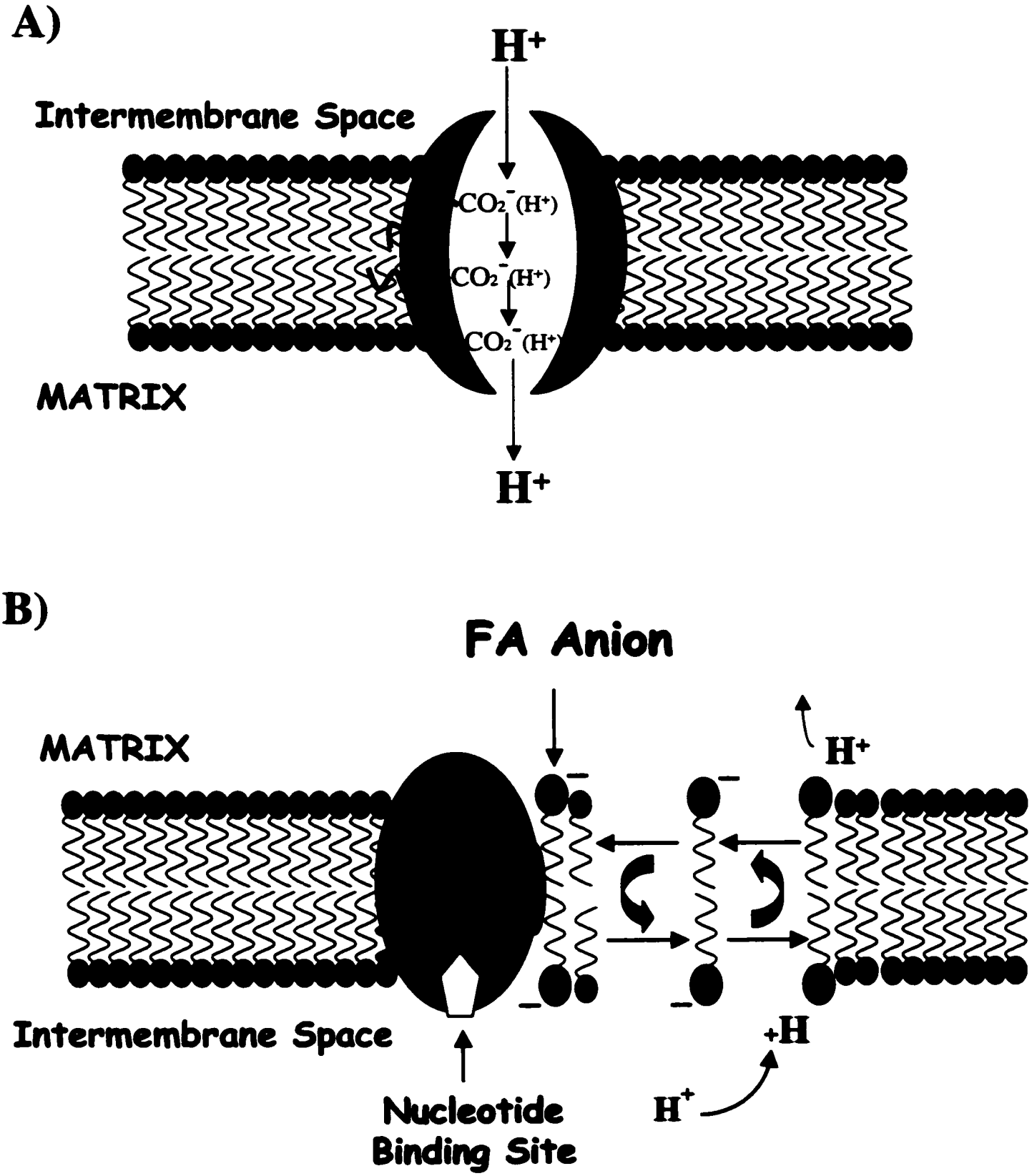
1.10.2 Fatty Acid Stimulation of UCP1-mediated Proton Leak: Two Different Points of View

It has been recognized for some time that the presence of fatty acids is necessary for

UCP1 acitivity. This was first inferred from various studies with mitochondria (Locke and Nicholls, 1981; Locke *et al.*, 1982; Rial *et al.*, 1983). Although UCP1 can be activated by fatty acids of various chain lengths, peak activation occurs with laurate (C_{12}) and myristate (C_{14}), while more commonly used palmitate (C_{16}) is less effective (Klingenberg and Huang, 1999). The mechanism of activation of uncoupling by fatty acids (FAs), however, it still one of the most debated issues in the uncoupling protein research community. There are two proposals, either fatty acids are the translocated species, or they act as cofactors.

Two distinct and controversial hypothetical models which have been put forth to attempt to explain how H^+ transport occurs via UCP1, and is affected by FAs. One model, commonly referred to as the *'fatty acid buffering model'* was introduced by Martin Klingenberg and his colleagues (Klingenberg, 1990; Klingenberg, 1999; Klingenberg and Huang, 1999). Klingenberg proposes that protons move through an aqueous pathway in UCP1, and that fatty acid carboxyl groups are lined up along the pathway as buffering cofactors that operate in conjunction with resident proton-conducting amino acids, such as histidines (Figure 3a). These donor/acceptor groups within the translocation channel are mostly carboxyl groups which enable unidirectional H^+ transport by providing a pK_a gradient. In this model the fatty acid molecules do not actually traverse the membrane bilayer, and are thought to be in equilibrium with FAs in the lipophilic phase of the mitochondrial inner membrane (Klingenberg and Huang, 1999). Klingenberg's group also demonstrated that manipulation of residues in UCP1 via mutagenesis can result in the inhibition of proton transport in UCP1. A pair of histidines (H145 and H147), on the matrix side near the centre of the sequence was found to be essential for H^+ transport in UCP1

FIGURE 3. TWO MODELS OF UCP-MEDIATED UNCOUPLING OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION. (A) THE KLINGENBERG 'FATTY ACID BUFFERING' MODEL (B) THE SKULACHEV/GARLID 'FATTY ACID FLIP-FLOP' MODEL.


A)
H+
Intermembrane Space
CO2- (H+)
CO2- (H+)
CO2- (H+)
MATRIX
H+
B)
FA Anion
MATRIX
H+
Intermembrane Space
+H
H+
Nucleotide
Binding Site

(Bienengraeber *et al.*, 1998). Although less specific for UCP, helix terminating D195 and D233 were also found to be important for H^+ transport.

The second proposed mechanism of action of FAs on UCP1 is the *'protonophoretic cycle'* or *'fatty acid flip-flop'* model that was introduced by Vladimir Skulachev (Skulachev, 1998) and that has been extensively studied by Keith Garlid and coworkers (Garlid, 1990; Garlid *et al.*, 1998; Garlid *et al.*, 2000; Garlid *et al.*, 1996). Here, UCP1 is thought to catalyse a flip-flopping of the anionic head group of fatty acids from the inner to outer leaflet of the mitochondrial inner membrane (Figure 3b). Transport of the anion is driven by the high inside-negative membrane potential. After the carboxylic acid head group has crossed the membrane, it picks up a proton, and the protonated fatty acid spontaneously flip-flops back to the matrix side, where the cycle begins all over again. Thus, in this model protonated fatty acids do not actually go through UCP1, rather they are thought to associate with an energy pore/well on the subsurface of the uncoupling protein.

1.10.3 Fatty Acid Effects on Mitochondria and Proteoliposomes

When reconstituted from BAT or yeast mitochondria, UCP1 requires exogenous fatty acids for its activity (Ricquier *et al.*, 1992). Using bacterially expressed and reconstituted UCP2 and UCP3, fatty acids either have no effect on stimulating proton conductance (Echtay *et al.*, 1999) or, as with UCP1 reconstituted from BAT or yeast, are required in order to observe this activity (Jaburek *et al.*, 1999). In the latter study, different fatty acid preferences are indicated between UCP2 and UCP3. In contrast to UCP1-expressing yeast, UCP2 and UCP3 respiration is not stimulated by 2-bromopalmitate or

palmitate (Hagen *et al.*, 2000). A recent study by Echtay and coworkers has suggested that perhaps the reason studies attempting to demonstrate fatty acid-mediated proton conductance by UCP2 and UCP3 are unsuccessful is because they lack the presence of a necessary cofactor. They eloquently showed that in the presence of coenzyme Q (CoQ, ubiquinone), fatty acids can indeed activate proton transport in *Escherichia coli* inclusion bodies expressing hUCP2 and hUCP3 (Echtay *et al.*, 2001).

1.10.4. Role of Retinoids in proton transport by UCP1 and UCP2

Retinoic acid is a proven powerful activator of the transcription of the Ucp1 gene (Alvarez *et al.*, 1995; Cassard-Doulcier *et al.*, 1994). It was only recently that the effect of retinoic acid on UCP1 protein was investigated (Rial *et al.*, 1999). The results showed that yeast mitochondria expressing UCP1 display higher affinity for all-*trans* retinoic acid than palmitate although the maximum rate of transport is lower. The concentration needed for activation of the protein was close to that which is needed to increase transcription of Ucp1. This pathway may be of physiological relevance since adipocytes play an active role in the metabolism of retinoids. In the same study, UCP2 was also shown to be activated by all-*trans* retinoic acid although the conditions required to observe uncoupling differed from those in UCP1. All-*trans* retinoic acid had no effect on UCP3.

1.11. Further Studies into Mouse Models of UCP Function

The overall objective of the research presented herein is to further examine mitochondrial proton leak and UCP expression, specifically UCP3, in rodent models and

human obesity. The models chosen for this research are the *Ucp3*-knockout mice, mice overexpressing UCP3, and a distinct human population.

To assess the metabolic significance and control of the proton leak we have used top-down elasticity analysis as our theoretical platform. This will be described in detail in *Section 1.12.*

1.11.1 The UCP3-Knockout Mouse

As mentioned above, the *Ucp3*-knockout mouse has normal serum insulin, triglyceride, and leptin levels, with a tendency toward reduction in free fatty acids and glucose (Gong *et al.*, 2000; Vidal-Puig *et al.*, 2000). Mice lacking the protein were found to have normal circadian rhythms in body temperature and motor activity and had normal body temperature responses to fasting, stress, thyroid hormone, and cold exposure (Gong *et al.*, 2000). The objective of my studies with these mice was to investigate the characteristics of the mitochondrial proton leak in the context of oxidative phosphorylation in mitochondria isolated from skeletal muscle, brown adipose tissue, and liver. In addition, we set out to determine whether various free fatty acids could stimulate proton conductance in control mitochondria, and if this activation was absent in mitochondria lacking UCP3.

1.11.2 Mice Overexpressing UCP3

Our collaboration with Dr. John Clapham at SmithKline-Beecham afforded us access to the newly created strain of mice overexpressing UCP3 (UCP-3Tg mice). As mentioned, UCP-3Tg mice overexpress human Ucp3 in their skeletal muscle, are hyperphagic, but are

significantly more lean than their wild-type littermates, and exhibit lower fasting plasma glucose and insulin levels. These observations point to UCP3 having the potential to influence metabolic rate and glucose homeostasis. The objective of my studies was to isolate mitochondria from various tissues in these mice and analyse the kinetics of the proton leak in order to determine whether the lean phenotype observed in these mice can be explained by an increased mitochondrial proton conductance. This was done in addition to immunoblotting of the mitochondrial proteins, as well histology of various tissues. These studies generated important new data on these mice, and impart greater insight into the putative role of the UCP homologues.

1.11.3 Links Between UCPs and Energy Expenditure and Obesity in Humans

The Ottawa Hospital Weight Management Program uses a modified Optifast® program and is a medically supervised program used in the treatment of patients who are obese and who may have significant medical problems related to obesity. This weight management program, directed by Dr. Robert Dent, has been quite a success. Over 2000 patients have participated in the program, and many more have received a referral from their primary care physician and are waiting to become admitted into the program. Essentially, the program involves the patient following an 8 month (BMI 30-37) or a 12 month (BMI > 34) standardized 900 kcal/day diet. In addition, patients are expected to attend weekly interactive teaching sessions which aim to instruct them on healthy eating habits, exercise regimes,...etc. Throughout the duration of the program, lipid profiles of patients and other clinical parameters are critically assessed.

The patients in the Weight Management Program are tightly monitored. An interesting finding was that despite an equivalent intake of 900 kcal/day diet, there existed a 3-fold difference in the rate of weight loss in healthy and highly compliant women, closely matched for age, and initial weight (Dent *et al.*, 1999). Patients were excluded from the study if they *a)* were being treated with medication that could affect weight loss (*e.g.*, lithium, tricyclic antidepressants, glucocorticoids, fenfluramine,...etc), *b)* had medical conditions which could affect the rate of weight loss including: congestive heart failure, sleep apnea, diabetes mellitus treated with insulin or other agents, hypothyroidism,...etc, *c)* smoked, or *d)* had impaired compliance scores. Selection of compliant patients was maximized by studying patients **in the first 6 weeks** of an 8 or 12 month weight management program with substantial cost to the patient ($1000 to $1700 USD) and by excluding those who did not meet the compliance criteria (See Material and Methods section 2.4.2. for details of criteria). Statistical analyses revealed that initial weight and age explained 30% of the variability in weight loss, and 35% of the variability in body fat loss. Initial serum free T_3 concentration could only explain 5% of the weight loss. Thus, two-thirds of the variability in weight loss was left unexplained by factors known to regulate energy requirements.

The overall goal of our studies with these patients was to determine metabolic, genetic and biochemical determinants of weight loss in response to an energy restricted diet. More specifically, our objective was to examine the role of UCP3 and mitochondrial proton leak in patients on the Optifast program who were identified as either *diet-reponsive or diet-resistant* individuals. The hypothesis has been that genetically based differences in Ucp3,

and differences in mitochondrial energetics could determine efficiency of energy substrate conversion to cellular ATP and hence explain the differences in an individuals ability to lose weight. In addition to detecting differences in levels of Ucp3 mRNA and UCP protein between *fast* and *slow* losers, our extended research group was involved in determining whether polymorphisms in Ucp3 exist in our study subjects, and could be potential predictors of rate of weight loss and resting metabolic rate.

1.12 Assessment of Proton Leak Kinetics via the Top-Down Elasticity Approach

Top-down elasticity analysis is an a extension of metabolic control analysis, an approach originally developed by Kacser and Burns (Kacser andBurns, 1973), and Heinrich and Rapoport (Heinrich and Rapoport, 1973), and reviewed extensively by others (Brand and Murphy, 1987; Fell, 1992; Kacser and Porteous, 1987). It is an approach that is of both theoretical and practical use for determining quantitatively the control structure of a metabolic system. Metabolic control analyses provide quantitative information about the importance of reactions, or blocks of reactions, in the control of flux (or rate of reactions) through a metabolic pathway, and concentration of metabolites within that pathway. This information is mathematically conveyed in the form of flux control and concentration control coefficients, respectively (Brown *et al.*, 1990a; Hafner, 1990). There are two approaches which can be taken when analysing a system using metabolic control analysis: the traditional approach, or the top-down approach. The aim of the former approach is to determine the flux control coefficients of *individual* enzymes of a metabolic pathway. By repeatedly applying this approach, the flux control coefficients of all the enzymes of each pathway of

the system can be determined. The applicability of this approach is limited, however, since it is restricted to particular enzymes and systems because of the necessity for, and limited availability of, specific inhibitors for enzymes acting within the system being studied (Harper and Brand, 1995).

The basic premise of this approach is that the control structure of a metabolic system is determined for blocks of enzymatic reactions, rather than for individual reactions. This further emphasizes the idea that control may be distributed throughout a system rather than being located at one particular site such as in a 'rate-limiting step'. The top-down approach involves conceptually dividing a metabolic pathway into two or three blocks of reactions around one of its intermediates. The intermediate chosen must be a component of the system which is produced by one or more pathway(s) and is consumed by subsequent pathway(s). There are, however, requirements which must be met before the top-down approach can be applied. First, the intermediate measured must be representative of the intermediate that the system actually uses. Thus, the intermediate cannot be channelled, compartmented, or diffusion limited. The second requirement is that the blocks of reactions affect each other solely through the concentration of the intermediate. In other words, there can be no 'cross talk' between the pathways within the system being studied.

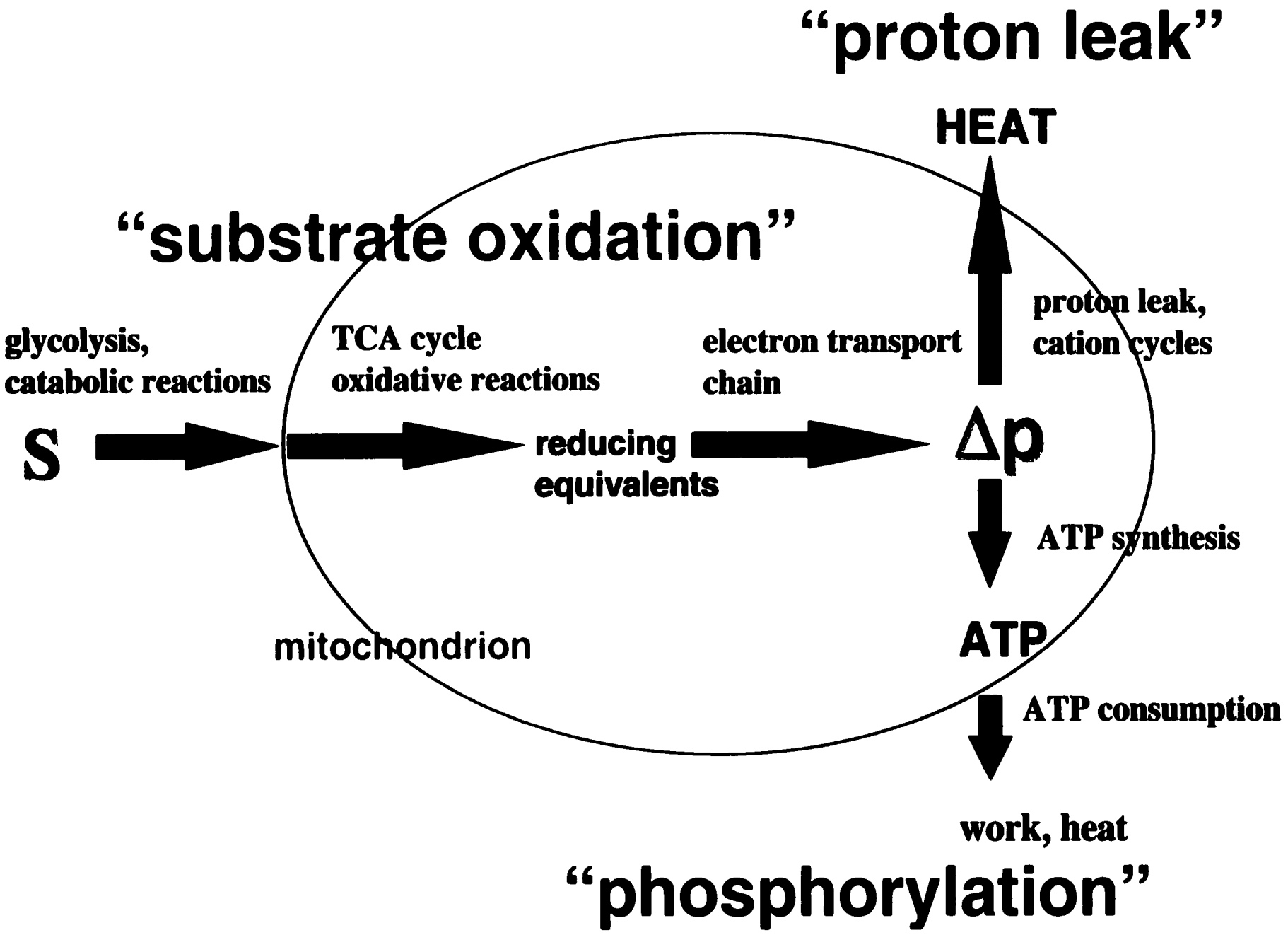
While metabolic control analysis allows the identification and quantitative description of important sites of control within metabolic pathways, the top-down elasticity approach allows for the identification of the sites of regulation within metabolic pathways. Elasticity analysis can be valuable in identifying the sites of metabolic effects of the insertion of a transgene, of gene knockout, or of the treatment with hormones, or drugs. Top-down

elasticity has been proven useful in elucidating the sites of action of glucagon (Brand *et al.*, 1990), thyroid hormones (Hafner *et al.*, 1990; Hafner *et al.*, 1988; Harper and Brand, 1994), and of fatty acids in isolated hepatocytes of rats (Nobes *et al.*, 1990b). Top-down elasticity analysis is an extension of metabolic control analysis and the data generated by this approach provides all of the information needed to complete a control analysis. Top-down elasticity analysis, thus allows for the determination of distribution of control between two or three distinct blocks of reactions comprising a system (Brown *et al.*, 1990a; Hafner, 1990).

At a practical level, to determine the distribution of control within the system being studied, the concentration of the intermediate is manipulated by titrating the blocks of reactions with various inhibitors or activators. The fractional change in the flux through the block of reactions that is caused by an infinitesimal fractional change in the concentration of the intermediate is referred to as the *elasticity* (*i.e.*, overall kinetics) of that block of reactions (Harper and Brand, 1995). The metabolic system which is the focus of the work presented in this thesis is the oxidative phosphorylation system. Top-down elasticity has been used to determine how oxidative phosphorylation is regulated. For our investigations, we have defined the oxidative phosphorylation as the tripartite system shown in Figure 4. Here, Δp is an appropriate intermediate since it is a component of the system that is produced by one pathway (electron transport chain reactions), and consumed by subsequent pathways (*i.e.*, ATP synthesis ands the mitochondrial proton leak). By manipulating the concentration of the intermediate (Δp) by titrating with various inhibitors and activators of the blocks of reactions, and simultaneously measuring flux rate through the pathway (oxygen consumption), distribution of control, or in this case, sites of regulation within the system

can be determined.

FIGURE 4. SCHEMATIC REPRESENTATION OF THE BRANCHED OXIDATIVE PHOSPHORYLATION SYSTEM. The intermediate within the system, the mitochondrial protonmotive force (Δp), is produced by the electron transport chain and consumed by both the phosphorylation reactions and the proton leak reactions. The proton leak reactions consists of the leakage of protons and any cation cycles across the mitochondrial inner membrane. The phosphorylating subsystem includes Δp-dependent ATP synthesis and all cellular ATP-consuming reactions.


"proton leak"
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proton leak,
cation cycles
glycolysis,
catabolic reactions
TCA cycle
oxidative reactions
electron transport
chain
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reducing
equivalents
Δp
ATP synthesis
mitochondrion
ATP
ATP consumption
work, heat
"phosphorylation"

2. METHODS AND MATERIALS

2.1 GENERAL

2.1.1 DEFATTING OF BOVINE SERUM ALBUMIN (BSA)

Defatted BSA is used to adsorb fatty acids in several of the solutions for the isolation and incubation of mitochondria. BSA was defatted by method of Chen (Chen, 1967). 50 g of BSA (fraction V, Sigma®) was dissolved in 250 ml of ddH_2O. After approximately 1 h of magnetic stirring, 25 g of activated charcoal (acid washed, BDH®), suspended in 100 ml of ddH_2O, was added. The suspension was then brought to a pH of 3.0 with 10 M HCl and stirred on ice for 1 h. Using a Beckman® J2-21M centrifuge with a JA 14 rotor, the suspension was spun at 11,300 rpm (20,000*g*) for 20 min at 4°C. The supernatant was carefully poured off into a 500 ml beaker, slowly brought to a pH of 7.0 with 10 M NaOH, and the spin was repeated. The resulting supernatant was filtered through a vacuum filtration apparatus fitted with a 0.45 um filter membrane (Millipore® HA type) and then through a 0.22 um filter membrane (GS type). Filtrate was dialysed against 153 mM NaCl and 10.8 mM KCl three times; twice for 1h and once overnight at 4°C. Prior to dialysis, the dialysis tubing was boiled in 75 mM ethylenediamine tetraacetic acid (Na_2EDTA; disodium salt) for 5 min and then in ddH_2O for an additional 5 min. Then 300 ml of the last dialysis medium was kept for dilutions. The concentration of defatted BSA was determined using a modified Lowry method (Lowry *et al.*, 1951), described below. The suspension was diluted in order to give a final stock concentration of 9% BSA (w/v) and then stored at -20°C in 10 ml aliquots for later additions to media and for making BSA working standard solutions for protein determinations.

2.1.2 Lowry Determination of Protein Concentration

Protein concentration of the mitochondrial suspension was assayed using a modified Lowry method (Lowry *et al.*, 1951) using various concentrations of a BSA standard to create a standard curve. 5 µl of mitochondrial suspension was added to a test tube containing 5 µl of mitochondrial isolation medium and 990 µl sodium hydroxide (0.5 N). Protein standards were prepared using a 30 µg protein/ml 0.5 N NaOH working BSA standard (0.3 ml stock BSA (10 mg/ml H_2O)) as outlined in the table below:

Protein Concentration (µg)	Working Standard (µl)	Isolation Medium (µl)	0.5 N NaOH (µl)	Copper Reagent [1] (ml)	Phenol Reagent [2] (ml)
0	0	10	990	1	4
15	50	10	940	1	4
30	100	10	890	1	4
60	200	10	790	1	4
90	300	10	690	1	4
120	400	10	590	1	4

Isolation medium, NaOH, and copper reagent were mixed first and allowed to sit at room temperature for 10 min. Immediately thereafter, phenol reagent was added and the solution was well mixed. All tubes, including the sample tube, were then incubated at 55°C for 5 min. The tubes were then placed on ice for 10 min in order to stop the reaction. Absorbencies

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[1] 10% Na_2CO_3, 0.1% $K_2C_4H_4O_6 \cdot ½ H_2O$, 0.05% $CuSO_4 \cdot 5 H_2O$

[2] Diluted 2N phenol reagent (Folin and Ciocalteu) 1:16 in H_2O just before use

were read at 550 nm on a Beckman DU-50 spectrophotometer. BSA standard curves were constructed by plotting absorbance as a function of μg protein and the protein concentration of the mitochondrial suspension was subsequently calculated.

2.1.3 UCP Detection by Western Blotting

2.1.3.1 Preparation of Samples

UCP was detected in isolated mitochondria using polyacrylamide gel electrophoresis (PAGE) and Western blotting. On the day of the assay, a volume of mitochondria containing 40 μg of protein was combined with an equal volume of sample loading buffer (2% SDS, 0.1 M Tris-HCl, 4 mM PMSF in isopropanol, 2 mM EDTA with bromophenol, 25% glycerol (w/v), and 10% β-mercaptoethanol (v/v). Along with the samples, we loaded Rainbow™ coloured protein molecular weight markers (Amersham Pharmacia Biotech®), Santa Cruz® biotinylated molecular weight markers, and mouse recombinant UCP3 blotting standard (0.25 μg; Stratagene®). The Rainbow™ markers were prepared by combining them 2:3:5 with PBS (phosphate buffered saline; 1.1 mM K_2HPO_4, 0.3 mM KH_2PO_4, 28 mM NaCl, 0.6 mM KCl; pH 7.4) and sample loading buffer. Before being loaded on the gel, samples, Cruz® MW markers, and recombinant UCP3 were boiled for approximately 3 min; Rainbow™ markers were boiled for 1 min.

2.1.3.2 Resolution of Mitochondrial Protein Samples by SDS-PAGE

For casting and running the gels, the Protean II Mini Electrophoresis® Cell (Bio-Rad®) was used. The separation gel (16%) used consisted of 10% acrylamide, 0.13% bis-acrylamide,

9% Tris-base, 0.1% SDS, and 0.1% ammonium persulfate. After the gel was poured, it was overlayed with distilled water in order to prevent drying, and left to polymerize for 1 hr at room temperature.

Following polymerization, the water was poured off by inversion of the assembly apparatus, a 4% stacking gel (3.4% acrylamide, 0.05% bis-acrylamide, 0.8% tris base, 0.1% SDS, and 0.1% ammonium persulfate) was poured on top of the separation gel. Combs were inserted and the gel was left to polymerize for 30 min at room temperature. After polymerization, combs were carefully removed, and the gels were placed in the electrophoresis cell. Running buffer (0.3% Tris base, 1.42% glycine, and 0.1% SDS) was added to the chamber and the samples were loaded using a Hamilton® syringe, and electrophoresis ran for 3 hrs at 180 V.

Once the electrophoresis was completed, the gels were removed from between the glass plates and placed in transfer buffer (0.72% glycine, 0.15% Tris base, and 20% methanol (v/v)) to equilibrate for 30 min at room temperature with gentle shaking. For transferring the proteins, a Mini Trans-Blot® Cell (Bio-Rad®) was used. The gels were set up in the transfer cassettes as follows: sponge fiber pad, 2 pieces of filter paper, equilibrated gel, nitrocellulose membrane (0.45 micron, Bio-Rad), 2 pieces of filter paper (Whatman® grade No.1), sponge fiber pad. Each component of the transfer was pre-soaked in transfer buffer for 30 min before assembly. The assembled cassette was placed into the Trans-blot cell with the nitrocellulose membrane facing the anode. An ice pack and a magnetic stir bar were placed in the chamber, and the transfer was conducted at 120 V for exactly 1 hr. Once the transfer was complete, the nitrocellulose membrane (blot) was carefully separated from the filter paper, wrapped in

Fisher® all-purpose laboratory wrap (polyvinyl-chloride) and stored at 20°C until further processing (no more than two days after transfer).

2.1.3.3 Western Blotting with UCP Antibodies

On the day of the experiment, the blot was removed from its plastic wrap and placed in 100 ml of blocking solution (PBS, 0.1% (v/v) Tween-20 and 5% (w/v) skim milk powder) for 1 hr at room temperature with gentle shaking[1] on a Belly Dancer® Shaker (Stovall Life Science Inc.). After blocking, the membrane was washed in a solution containing PBS and 0.1% Tween-20 (v/v). Thereafter, there were two quick rinses, 2 five minute, and 1 ten minute washes, each with approximately 50 ml of washing solution (fresh solution between each wash) at room temperature with vigorous shaking[2] on the Belly Dancer®.

The washed membrane was incubated in a tube with 3 ml of a primary antibody (polyclonal rabbit anti-human uncoupling protein-3, Chemicon International Inc.), diluted 1:200 in antibody keeping solution (3% BSA and 0.2% NaN_3), for 1 hr at room temperature in a Labquake® rolling shaker (Labindustries Inc.). After incubation, washing steps as mentioned above were repeated with an extra 10 min wash. The membrane was then incubated with the secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, 200µg/0.5 ml, Santa Cruz Inc.), diluted 1:5,000 in PBS and 5% (w/v) skim milk powder solution for one hour at room temperature with gentle shaking. Washes were repeated as stated above, and an extra (*i.e.*, a third) 10 minute wash and two 5 min washes in 1x-PBS

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[1]Speed #4 on shaker
[2]Speed #7 on shaker

alone were performed.

Detection of secondary antibody conjugate was carried out using a ECL™ RPN 2109 Western blotting detection kit (Amersham Pharmacia Biotech®). This is a light emitting non-radioactive method for detection of immobilised specific antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. After taking the blot out of the PBS solution, it was laid flat in a large weigh boat, and its entire surface was covered with 1 ml of the ECL™ kit reagents (1ml Reagent #1 and 1 ml Reagent #2, mixed immediately before pouring). The blot was then left undisturbed for 1 min, after which time, the weigh boat was tipped and the excess solution was poured off. The blot was then placed face down in the camera dish and any air bubbles were carefully manually excluded.

Visualization of the blot was carried out by exposing it to Polaroid™ Polapan-667 black and white instant film using an Amersham® ECL Mini-camera. Exposure times varied from experiment to experiment. After the film was removed from the camera, it was left for 30 s before the film cover was removed.

2.2 STUDIES WITH UCP3-KNOCKOUT MICE

2.2.1 BROWN ADIPOSE TISSUE (BAT) MITOCHONDRIA OF UCP3 - KNOCKOUT MICE

2.2.1.1 TREATMENT OF ANIMALS

Male 4-6 months of age uncoupling protein 3-knockout (*Ucp3*-knockout) mice and male 129/SvJ wild-type controls (Gong *et al.*, 2000) were obtained from the research colonies of Dr. Mark Reitman at the Diabetes Branch, NIDDK, National Institutes of Health (Bethesda, Maryland). The mice were group housed (3/cage), given free access to Charles River 5075 rodent chow (4.5% fat by weight) and water, and kept at 23°C with light 07:00-19:00. Mice used in this study were cared for in accordance with the principles and guidelines of the Canada Council on Animal Care and the Institute of Laboratory Animal Resources (National Research Council, USA).

2.2.1.2 ISOLATION OF MITOCHONDRIA FROM BAT

Mitochondria were isolated from interscapular BAT depots of 6 UCP1-deficient and 6 control mice. Mice were killed by decapitation prior to removal of BAT. BAT was dissected free of other adhering tissues, weighed, and then homogenized manually in 3.5 ml of ice-cold buffer containing 250 mM sucrose, 1.0 mM HEPES, and 0.2 mM EDTA (pH 7.2 with KOH) using a glass/Teflon Potter-Elvehjem tissue grinder. For all centrifugations, a Sorvall® refrigerated centrifuge (RC2-B) was used with SS-34 rotor. Fractionation of the homogenate was carried out by spinning at 3,000 rpm (1,100*g*) for 10 min at 4°C. The supernatant was then poured through a 250 um filter (Nitex®) and respun at 10,000 rpm

(12,100*g*) for 14 min at 4°C to obtain a mitochondrial pellet. The pellet was resuspended (on ice) in 150 µl of a suspension medium containing 120 mM KCl, 20 mM sucrose, 3.0 mM HEPES, 2.0 mM $MgCl_2 \cdot H_2O$, 2.0 mM EGTA and 0.5% defatted BSA (pH 7.2 with KOH). Protein concentration of the mitochondrial suspension corrected for BSA in the medium was assayed using a modified Lowry protocol (see *Section 2.1.2*). As well as removing and weighing the interscapular BAT form the mice, epididymal and inguinal fat depots, were also removed and weighed.

2.2.1.3 Measurement of Mitochondrial Oxygen Consumption

The respiration of isolated BAT mitochondria was measured using a Hansatech® Clark-type oxygen electrode. The incubation chamber was maintained at 37°C and magnetically stirred. Each rate was assessed by incubating enough mitochondria in 1.0 ml of suspension medium to give approximately 0.50 mg mitochondrial protein/ml in the electrode chamber. All respiration rates were determined *simultaneously and in parallel with measurements of protonmotive force* in the chamber. Titrations were performed in the presence of 80 ng/ml nigericin in order to bring -zΔpH close to zero and effectively convert it to millivolt units (Brand, 1995). 5.0 µM of rotenone was added to prevent the oxidation of any endogenous NAD-linked substrates. *State 3* respiration rate was defined as the oxygen consumption rate in the presence of the complex II substrate, succinate (10 mM), in addition to ADP (10 mM), 0.65 units/ml hexokinase and 0.05µM FCCP. *State 4* oxygen consumption was determined in the presence of maximal amounts of the ATP synthase inhibitor, oligomycin (6 µg /mg mitochondrial protein). It was confirmed that ATP synthase was

completely inhibited in each experimental condition by additional oligomycin which caused no further inhibition of oxygen consumption and no further increase in protonmotive force. Mitochondrial proton leak activity was assessed in the presence of saturating amounts of oligomycin, with incremental additions of the respiratory chain inhibitor, malonate (0.33, 0.66, 1.0, 2.0, 5.0 mM).

2.2.1.4 MEASUREMENT OF MITOCHONDRIAL PROTONMOTIVE FORCE (Δp)

Δp was measured using a lipophilic organic ion probe, triphenylmethylphosphonium ($TPMP^+$). The accumulation of the probe was measured usin a $TPMP^+$-sensitive electrode (Kwik-Tip™) which was constructed by using the methods of Kamo (Kamo *et al.*, 1979). The outputs from the $TPMP^+$ electrode and the O_2 electrode were transferred to two voltmeters whose reference sockets were connected together; data were then fed into a data analysis software package (Duo 18™ data recording system) which allowed real-time monitoring and recording of data on a personal computer. The calibration of the $TPMP^+$-sensitive electrode, determination of mitochondrial matrix volumes, measurement of non-specific binding in mitochondria (a_m), and calculation of Δp from $TPMP^+$ electrode data were carried out as outlined below. Δp was calculated using the Nernst equation as:

$$\Delta p = 61.5 \cdot \log (a_m \cdot TPMP^+_m / TPMP^+_e)$$

$TPMP^+_m/TPMP^+_e$ represents the ratio of the accumulation of the cation inside and external to the mitochondria (See *Section 2.2.1.6*).

2.2.1.5 CALIBRATION OF $TPMP^+$-SENSITIVE ELECTRODES

Prior to each titration the $TPMP^+$ electrode was calibrated. Mitochondria (0.5 mg protein/ml) were added to the electrode chamber which contained 1.0 ml of suspension medium, nigericin (80 ng/mg protein) and rotenone (5.0 μM). The $TPMP^+$ electrode was then inserted and the incubation chamber sealed; once the trace was steady, TPMP (10μM) was added. When the trace reached a new steady state value (5-20 s) a second 10 μM aliquot of $TPMP^+$ was added and another new steady state achieved. These additions were repeated until the total final $TPMP^+$ concentration was 50 μM. Succinate (10 uM) was then added and mitochondria were allowed to accumulate the $TPMP^+$ until an equilibrium distribution was achieved and the extramitochondrial $TPMP^+$ concentration was stable (~ 1 min). Subsequent additions of various inhibitors, ionophores, or other compounds were then made, with the new steady state values being obtained within approximately one minute of each addition.

2.2.1.6 CALCULATION OF ΔP FROM $TPMP^+$ ELECTRODE DATA

The deflection caused by each 10 uM $TPMP^+$ addition in chart units from the baseline recorded was measured and plotted against the logarithm of the final $TPMP^+$ concentration to produce a calibration graph. To measure the external $TPMP^+$ concentration, $TPMP^+_e$, for any given electrode signal, the deflection from the baseline (in chart units) to the new steady state was determined. $TPMP^+_e$ was then directly read off of the calibration graph. The concentration of $TPMP^+$ in the mitochondrial matrix ($TPMP^+_m$) was determined as follows:

$$TPMP^{+}_{m} = \frac{[TPMP]_{added} - [TPMP_c]}{(0.001 \cdot MV \cdot \text{mg protein/ml})}$$

where MV represents mitochondrial matrix volume (in ml). $TPMP^{+}_{m}$ and $TPMP^{+}_{e}$ were determined for each mitochondrial titration and used to calculate Δp.

2.2.1.7 Measurement of Mitochondrial Matrix Volume (MV)

When mitochondria are incubated with a radiolabelled probe such as $^{3}H_2O$ and then sedimented, the accessible volume of the pellet can be calculated from the specific activity of the probe in the supernatant and the total radioactivity in the pellet. The difference in accessible volume ('pellet space') for a permeant probes like H_2O and probes like [C^{14}]sucrose that do not cross the inner membrane reports the volume of the matrix. 40 µl of mitochondria (~2 mg protein) were incubated in 1.0 ml of suspension medium containing 5.0 µM rotenone, 1.0 µCi of $^{3}H_2O$ and 0.1 µCi of [^{14}C]sucrose, for 2 min in a 1.5 ml minitube maintained at 37°C in a water bath. Mitochondria were then sedimented by centrifugation at 12,000 *g* for 1 min in a Eppendorf® minifuge. 500 µl supernatant was removed and added to 7.5 ml of scintillation cocktail (Formula-989 Universal High Flash-Point LSC, Packard Bioscience B.V.). The remainder of the supernatant was decanted and the walls and cap of the tube carefully dried. Resuspension of pellet was carried out with 40 µl of 20% (v/v) Triton X-100 and by vortex mixing. Once the pellet was completely resuspended, the tip of the tube containing the mitochondrial pellet was removed and placed in 7.5 ml of scintillation cocktail. The radioactivity of the supernatant and pellet were determined by dual-channel

scintillation counting for ^{3}H and ^{14}C using the appropriate quench and cross-over corrections. Mitochondrial matrix volume (MV) was calculated as:

$$MV = \frac{(^{3}H_2O \text{ space} - [^{14}C]\text{sucrose space})}{\text{mg protein}}$$

Average mitochondrial matrix volumes were 0.85 µl/mg protein (± 0.15) in *Ucp3*-knockout mice and 0.45 µl/mg protein (± 0.20) in controls. These values were based on 6 determinations completed on BAT mitochondria isolated and pooled from 5 *Ucp3*-knockout and 5 control mice.

2.2.1.8 Measurement of Non-Specific Binding of $TPMP^{+}$ (A_m)

Non-specific binding (a_m) of the TPMP ion was determined as described by Nobes *et al.* (Nobes *et al.*, 1990b). $TPMP^{+}$ accumulation ratios and $^{86}Rb^{+}$ accumulation ratios in the presence of valinomycin were determined at three concentrations of KCl (0.2, 1.0 or 5.0 mM). For each concentration of KCl, two sets of minitubes were prepared; each set contained 80 µl of mitochondria (~2 mg protein), 1.0 ml of incubation medium (200 mM sucrose, 5.0 mM HEPES, 5.0 mM LiCl, 1.0 mM EGTA, 5.0 µM rotenone, 1.0 µM TPMP-Br, and 0.2 µg/ml valinomycin. To one set 0.035 µCi $^{86}RbCl$/ml and 0.15 µCi [^{3}H]TPMP/ml was added, and to the other 1.5 µCi $^{3}H_2O$/ml and 0.15 µCi [^{14}C]sucrose/ml was added. In each set, each concentration of KCl was assessed in duplicate. Succinate (5.0 mM; pH 7.0 with LiOH) was added to all tubes and they were mixed by inversion. After 2 min at room temperature the mitochondria were sedimented by centrifugation for 2 min at 12,000 *g*. 500

µl aliquots of the supernatant were removed and pipetted into scintillation vials containing 7.5 ml of scintillation cocktail. The residual supernatant was aspirated; the sides and cap were wiped dry and 40 µl of Triton X-100 was added. Following the complete suspension of the pellet by vortex mixing, the bottom of the tube was cut off into a scintillation vial and pellet was resuspended in 7.5 ml of scintillation cocktail containing 150 µl of ddH_2O. The radioactivity of the supernatant and pellet were determined by dual-channel scintillation counting for ^{3}H, ^{14}C and ^{86}Rb using the appropriate quench and cross-over corrections.

The apparent volume of pellet available to each isotope (in µl) was calculated as dpm in total pellet divided by dpm/µl of supernatant sample. The [^{3}H]TPMP^{+} accumulation ratio, $([TPMP^+]_{tot}/[TPMP^+]_e$, was calculated as ([$^{3}H$]TPMP^{+} space - [$^{14}C$]sucrose space)/($^{3}H_2O$ space - [^{14}C]sucrose space). The $^{86}Rb^+$ accumulation ratio, $[^{86}Rb^+]_{tot}/[^{86}Rb^+]_e$ was calculated as ($^{86}Rb^+$ space - [^{14}C]sucrose space)/($^{3}H_2O$ space - [^{14}C]sucrose space). By plotting [^{3}H]TPMP^{+} accumulation ratios against $^{86}Rb^+$ accumulation, a linear relationship is obtained. The inverse slope of this line is defined as a_m. Values for a_m were determined to be 0.25 and 0.20 respectively for *Ucp3*-knockout mice and controls. These mean values were based on data from BAT mitochondria isolated and pooled from 10 *Ucp3*-knockout and 10 control mice. It was necessary to pool the mitochondria within the two groups due to relatively large concentrations of mitochondria required for the accurate determination of a_m. As mitochondria needed to be pooled for each group, no SEM values are given.

2.2.1.9 Application of Top-Down Elasticity Analysis and Top-Down Control Analysis

To quantitatively determine the effects of the absence of Ucp3 on oxidative

phosphorylation processes in BAT mitochondria we used the top-down elasticity approach described by Brand (Brand, 1990b; Brand, 1998) and Harper and Brand (HarperandBrand, 1995). We defined the oxidative phosphorylation system as the tripartite system shown in Figure 4. The overall elasticities to changes in Δp of the reactions that produce Δp (*i.e.*, electron transport chain reaction) and the two blocks of reactions that consume Δp (*i.e.*, ATP synthesis and the mitochondrial proton leak reactions), were then determined.

The kinetic response of the leak to Δp was assessed by completely inhibiting proton return through ATP synthase using maximal amounts of the specific inhibitor oligomycin (6 μg/mg mitochondrial protein) and titrating with malonate (0.33-5.0 mM), a competitive inhibitor of complex II of the respiratory chain. Using this top-down approach, we are able to graph oxygen consumption as function of protonmotive force and examine the kinetics of the leak.

2.2.2 Skeletal Muscle Mitochondria of UCP3-Knockout Mice

2.2.2.1 Treatment of Animals

8 male (4-6 mo.) *Ucp3*-knockout mice and 8 male (4-6 mo.) 129/SvJ wild type controls were obtained from the research colonies of Dr. Mark Reitman at The Diabetes Branch, NIDDK, National Institutes of Health (Bethesda, Maryland). The mice were group housed (3/cage), given free access to Charles River 5075 rodent chow (4.5% fat by weight) and water, and kept at 23°C with light 07:00-19:00. Mice used in this study were cared for in accordance with the principles and guidelines of the Canada Council on Animal Care and the Institute of Laboratory Animal Resources (National Research Council, USA).

2.2.2.2 Isolation of Mitochondria from Skeletal Muscle

Mitochondria were isolated from hind limb and forelimb skeletal muscles of *Ucp3*-knockout and control mice. Specifically, these muscles included the muscles of the lower leg (gastrocnemius), thigh (vastus lateralis, rectus femoris, quadratus femoris, adductor brevis, semimembranosus, gluteus maximus, gluteus minimus, and gluteas medius), and shoulder (triceps longus and medius; biceps brevis and longus).

Mice were killed by decapitation prior to removal of skeletal muscle. Tissue was then immediately placed in ice-cold isolation medium (100 mM sucrose, 10 mM EDTA, 100 mM Tris-HCl, and 46 mM KCl; pH 7.4 with KOH). The muscle was dissected free of any visible connective tissue and fat. Clean pieces of muscle were placed on a pre-chilled plastic watchglass, carefully minced using safety razor blades and put back into isolation medium. Prior to homogenization, the tissue was filtered through 250 um filter and placed in 25 ml of isolation medium containing 5.0 mg of Nagarse (type XXVII protease, Sigma®) for 5 min at room temperature, with occasional stirring. Homogenization was performed using a cold glass/Teflon Potter-Elvehjem tissue grinder. Fractionation of the homogenate was carried out by centrifugation at 2,000 rpm (480*g*) for 10 min at 4°C in a Sorvall® RC2-B centrifuge with a SS-34 rotor. The supernatant was then poured through a 250 um filter into a clean polyethylene centrifuge tube and respun at 10,000 rpm (12,100*g*) for 10 min. The resulting supernatant was discarded and the pellet was resuspended using 5.0 ml of isolation medium containing 0.5% defatted BSA and respun at 10,000 rpm for a final 10 min. The resulting pellet was resuspended in 250 µl of suspension medium (120 mM KCl, 20 mM sucrose, 20 mM glucose, 10 mM KH_2PO_4, 5.0 mM HEPES, 2.0 mM $MgCl_2$ and 1.0 mM EGTA; pH 7.2

with KOH). Protein concentration of the mitochondrial suspension was assayed by the Lowry method (described in *Section 2.1.2*). At the time the mice were killed, brown adipose tissue, heart, epididymal and inguinal fat depots, were also removed and weighed.

2.2.2.3 MEAUSUREMENT OF MITOCHONDRIAL OXYGEN CONSUMPTION

The respiration of skeletal muscle mitochondria was measured using a Hansatech® Clark-type oxygen electrode whose incubation chamber was maintained at 37°C and magnetically stirred. Each rate was assessed by incubating enough mitochondria in 1.0 ml of suspension medium to give approximately 0.5 mg mitochondrial protein/ml in the electrode chamber. All respiration rates were determined simultaneously and in parallel with measurements of protonmotive force. Titrations were done in the presence of 40 ng nigericin/ml in order to convert -zΔpH to millivolt units, and in the presence of 5.0 μM of rotenone to prevent the oxidation of any endogenous NAD-linked substrates. *State 3* respiration rate was defined as the oxygen consumption rate in the presence of 10 mM succinate, 0.65 units/ml hexokinase, 100 μM ADP, 100 μM ATP, and 0.05 μM. *State 4* oxygen consumption was determined in the presence of saturating amounts of oligomycin (8 μg/mg mitochondrial protein). It was confirmed that ATP synthase was completely inhibited in each experimental condition by additional oligomycin which caused no further inhibition of oxygen consumption and no further increase in proton motive force. Mitochondrial proton leak activity was assessed in the presence of saturating amounts of oligomycin, with incremental additions of malonate (0.33, 0.66, 1.0, 2.0, 5.0 mM).

2.2.2.4 Measurement of Mitochondrial Protonmotive Force (Δp) and Calibration of $TPMP^+$ Electrodes

Measurement of Δp and calibration of $TPMP^+$-sensitive electrodes were carried out as described earlier in *Sections 2.2.1.4-6*

2.2.2.5 Measurement of Mitochondrial Matrix Volume

40 ul of mitochondria (~2 mg protein) were incubated in 1.0 ml of suspension medium containing 5.0 μM rotenone and 1.0 μCi of ^3H_2O, and 0.1 μCi of [^{14}C]sucrose, for 2 min in a 1.5 ml minitube maintained at 37°C in a water bath. Mitochondria were then sedimented by centrifugation at 12,000 *g* for 1 min. 500 μl supernatant was removed and added to 7.5 ml of scintillation cocktail. The remainder of the supernatant was decanted and the walls and cap carefully dried with a rolled-up tissue. Resuspension of pellet was carried out with 40 μl of 20%(v/v) Triton X-100 and by vortex mixing. Once the pellet was completely resuspended the tip of the tube was cut and placed in 7.5 ml of scintillant. The radioactivity of the supernatant and pellet were determined by dual-channel scintillation counting for ^{3}H and ^{14}C using the appropriate quench and cross-over corrections. Mitochondrial matrix volume (MV) was calculated as described in *Section 2.2.1.7.* Average mitochondrial matrix volumes were 0.52 μl/mg protein (± 0.16; n=2) in *Ucp3*-knockout mice and 0.48 μl/mg protein (± 0.18; n=2) in controls.

2.2.2.6 Measurement of Non-Specific Binding of $TPMP^+$ (A_m)

[^{3}H]$TPMP^+$ accumulation ratios and $^{86}Rb^+$ accumulation ratios in the presence of valinomycin were determined as described in *Section 2.2.1.8.* Values for a_m were determined

to be 0.31 and 0.36 respectively for *Ucp3*-knockout mice and controls. These mean values were based on data from skeletal muscle mitochondria isolated and pooled from 4 *Ucp3*-knockout and 4 control mice. Since our results are based upon one experiment, no SEM values were obtained.

2.2.2.7 Application of Top-Down Elasticity Analysis and Top-Down Control Analysis

See *Section 2.2.1.9*

2.2.2.8 Effects of free fatty acids on skeletal muscle mitochondria of UCP3-knockout mice

Mitochondria were isolated from 6 *Ucp3*-knockout and 6 control mice as described in *Section 2.2.2.2*. Oxygen consumption and mitochondrial protonmotive force were measured for each individual preparation of mitochondria as outlined in *Sections 2.2.2.3-6*. The effect of free fatty acids on the kinetics of the leak were determined by titrating mitochondrial incubations with saturating amounts of oligomycin (8 ug/ml) and increasing amounts of malonate (0.33-5.0 mM) in the presence of lauric acid, stearic acid, linoleic acid, or just vehicle (0.1% defatted) BSA. The estimated final concentrations of free fatty acids were: 66 μM of lauric acid, 27 μM of stearic acid, or 27 μM of linoleic acid.

2.3 Studies with mice overexpressing UCP3

2.3.1 Skeletal Muscle Mitochondria of Mice Overexpressing UCP3

2.3.1.1 Treatment of Animals

21 male (6 mo.) *Ucp3*-overexpression mice (UCP-3Tg) and 22 male (6 mo.)

C57BL/6J wild-type controls were obtained from the research colonies of Dr. John C. Clapham at SmithKline Beecham Pharmaceuticals (Harlow, United Kingdom). The mice were group housed (3/cage), given free access to Charles River 5075 rodent chow (4.5% fat by weight) and water, and kept at 23°C with light 07:00-19:00. Mice used in this study were cared for in accordance with the principles and guidelines of the Canada Council on Animal Care and the Institute of Laboratory Animal Resources (National Research Council, USA).

2.3.1.2 Isolation of Mitochondria from Skeletal Muscle

Skeletal muscle mitochondria were isolated from 9 UCP-3Tg mice and 9 control mice as outlined in *Section 2.2.2.2.* At the time that the mice were killed, brown adipose tissue, heart, epididymal and inguinal fat depots, were also removed and weighed.

2.3.1.3 Meausurement of Mitochondrial Oxygen Consumption

Mitochondrial oxygen consumption was determined as outlined in *Section 2.2.2.3.*

2.3.1.4 Measurement of Mitochondrial Protonmotive Force (Δp) and Calibration of TPMP$^+$electrodes

Measurement of Δp and calibration of $TPMP^+$-sensitive electrodes were carried out as described earlier in *Sections 2.2.2.4.*

2.3.1.5 Measurement of Mitochondrial Matrix Volume

Mitochondrial matrix volume (MV) was calculated as described in *Section 2.2.2.5.* Average mitochondrial matrix volumes were 0.79 μl/mg protein (± 0.16) in UCP-3Tg mice and 0.58 μl/mg protein (± 0.18) in controls.

2.3.1.6 Measurement of Non-Specific Binding of $TPMP^+$ (A_m)

[^{3}H]$TPMP^+$ accumulation ratios and $^{86}Rb^+$ accumulation ratios were determined as described in *Section 2.2.2.6.* Values for a_m were determined to be 0.47 and 0.33 respectively for mice overexpressing UCP3 and controls. These mean values were based on data from skeletal muscle mitochondria isolated and pooled from 3 UCP-3Tg and 3 control mice. Since our results are based upon one experiment, no SEM values were obtained.

2.3.1.7 Application of Top-Down Elasticity Analysis and Top-Down Control Analysis

See *Section 2.2.2.7.*

2.3.1.8 Histology: Thin Section Light Microscopy

Upon dissection, pieces of freshly cut tissue were weighed and then immediately placed in 10% buffered Formalin™. The tissues were left to fix in formalin for at least three days before being processed. All subsequent steps including embedding, preparation of slides, and staining were kindly performed by a technician in Dr. Jean Himms-Hagen's laboratory, Linda Jui

a)Paraffin Embedding of Sections:

The tissue pieces were placed in plastic cassettes (HISTOSETTE®, Simport Plastics). The tissue was then processed in an automated machine in the following manner: First the tissue was dehydrated using increasing amounts of ethanol (60, 70, 80, 90, 100%). The tissue was then washed twice with xylene, and finally with paraffin by hand.

The paraffin blocks were then sectioned into 4 μm slices, using a microtome (Tissue-

tek® '820' microtome, Spencer). These sections were then placed onto Superfrost Plus® slides.

b) Staining of Thin Sections:

The paraffin embedded sections were then deparaffinized and rehydrated by the following method. The sections were left in xylene for 5 minutes (twice), then washed in 100% ethanol for 3 minutes, once in 95% ethanol for 3 minutes, once in 70% ethanol for 3 minutes, and once in water for 5 minutes. For Haematoxylin and Eosin (H&E) staining, the sections were first incubated in filtered Delafield's Haematoxylin (Sigma) for 6 minutes, and then rinsed in water differentiated in alcohol (30 % alcohol + 1% HCl) for 3 seconds. The slides were then washed with lithium carbonate (1g/100 ml distilled water) until the section was blue. After a wash in running water for 10 minutes, the slides were placed in eosin (Sigma) for 2 minutes. They were then dehydrated once in 95% alcohol for 2 minutes, twice in 100% alcohol for 2 minutes, and once in xylol for 5 minutes. Finally, the sections were mounted on slides with Permount.

c) Viewing and Photography of Slides

The slides were viewed using a light microscope (Zeiss Axioskop 20, West Germany) under 400x magnification and were photographed using an attached camera. The film used was Kodak Ektachrome 64T (tungsten) Professional, Colour Reversal Film (EPY 135-36) and was taken to a commercial photographic shop for processing.

2.3.1.9 WESTERN BLOTTING ANALYSIS

See *Section 2.1.3* for details of protocol. Samples were run on each gel in the

following order: Rainbow™ coloured protein markers, Cruz® MW markers (Santa Cruz Inc.), recombinant UCP3, control mitochondria, mitochondria overexpressing UCP3, control mitochondria, mitochondria overexpressing UCP3, *Ucp3*-knockout mitochondria, Cruz® MW markers. Mitochondria isolated from control and UCP-3Tg mice studied on the same experimental day were run on the same gel.

2.4. Studies with Obese Diet-Resistant Women in the Ottawa Hospital Weight Management Program

2.4.1 Clinical Protocol

The study was approved by the Human Research Ethics Committee of the Ottawa Hospital. Rate of weight loss was evaluated in first six weeks of a 900kcal meal replacement component of a commercially available meal replacement program (Optifast 900®, Novartis Nutrition, Canada).

2.4.2 Selection of Study Population

Under the supervision of Dr. Robert Dent at the Ottawa Hospital-Civic Campus, approximately 1130 female subjects, ranging in age from 18 to 65, and with BMI 30-50 completed the Optifast® weight management program over a ten year period. Selection of compliant patients was maximized by studying them in the first 6 weeks of an 8 or 12 month program with substantial cost to the patient ($1600 to $2600 USD) and excluding those who did not meet the compliance criteria[1] or medical criteria[2]. After non-compliant patients were identified and excluded from our study, there remained 353 patients in the study group.

Patients who fell into the highest (71 subjects) or lowest quintile (70 subjects) of weight loss were invited to participate in our study. Twelve volunteers from of the upper and

---

[1]Excluded if: completed less than 75% of the 16 or 26 weekly visits, absent for more than 2 of the 6 visits in the study segment, inadequate completion of the lab testing protocol.

[2]Excluded if: TSH out of normal range, diabetes treated with insulin or oral hypoglycemics, smokers, surgery during study segment, obstructive sleep apnea, congestive heart failure, wheelchair-bound, previous surgery for weight control, cancer diagnosed at any time during program, use of tricyclic antidepressants at doses greater than 50 mg per day, or use and of the following: oral glucocorticoids, antipsychotics, beta-blockers, lithium, appetite suppressants or orlistat, fluoxetine at doses greater than 30 mg per day.

lower quintiles (diet-responsive and diet-resistant losers of weight, respectively) agreed to be involved in the study, and provided their informed consent.

2.4.3 STUDIES WITH SKELETAL MUSCLE

2.4.3.1 MUSCLE BIOPSIES

Muscle biopsies were performed on 12 diet-responsive and 12 diet-resistant women matched for initial weight and age, by plastic surgeon, Dr. Lloyd VanWyck. Subjects were fasting between 7 and 9 am. An open biopsy (~ 3g) of the rectus femoris was taken under local (lidocaine) anaesthesia. On each experiment day, one slow and one fast loser subject was sampled. Approximately 50 mg of tissue was immediately frozen and stored at -80°C for RNA analyses and the remaining portion was used for mitochondrial isolation.

Biopsies were performed several months [(22.1 ±4.1 mo (diet-responsive); 18.7 ±5.0 mo (diet-resistant) after meal replacement completion and after 4 weeks of weight stabilization.

2.4.3.2 ISOLATION OF SKELETAL MUSCLE MITOCHONDRIA

Biopsies were immediately placed in glass vials containing ice-cold isolation medium (100 mM sucrose, 10 mM EDTA, 100 mM Tris-HCl, and 46 mM KCl; pH 7.4 KOH) and the vials were kept on ice. The muscle was dissected free of any visible connective tissue and fat. Clean muscle depots were then carefully minced using safety razor blades and placed in 5 ml of isolation medium containing 0.5% defatted BSA. Prior to homogenization, the tissue was filtered through 250 um Nitex filter and placed in 25 ml of isolation medium containing

5.0 mg of Nagarse (type XXVII protease) for 2 min at room temperature, with occasional stirring. Homogenization was performed manually using a cold glass/Teflon Potter-Elvehjem tissue grinder. Fractionation of the homogenate was carried out by centrifugation at 2,000 rpm (480*g*) for 10 min at 4°C in a Sorvall® RC2-B centrifuge with a SS-34 rotor. The supernatant was then poured through a 250 um filter into a clean polyethylene centrifuge tube and respun at 10,000 rpm (12,100*g*) for 10 min. The resulting supernatant was discarded and the pellet was resuspended using 5.0 ml of isolation medium and centrifuged again at 10,000 rpm for a final 10 min. The resulting pellet was resuspended in 250 µl of suspension medium (120 mM KCl, 20 mM sucrose, 20 mM glucose, 10 mM KH_2PO_4, 5.0 mM HEPES, 2.0 mM $MgCl_2$ and 1.0 mM EGTA; pH 7.2 with KOH). Protein concentration of the mitochondrial suspension was assayed by the Lowry method (see *Section 2.1.2*).

2.4.3.3 Measurement of Mitochondrial Oxygen Consumption

As described in *Section 2.2.2.3*.

2.4.3.4 Measurement of Mitochondrial Protonmotive Force (Δp) and Calibration of TPMP⁺ Electrodes

Measurement of Δp and calibration of $TPMP^+$-sensitive electrodes were carried out as described earlier in *Sections 2.2.2.4*.

2.4.3.5 Western Blotting Analysis

See *Section 2.1.3* for details of protocol. Samples were run on each gel in the following order: rainbow markers, Cruz® MW markers, recombinant UCP3, diet-resistant A

mitochondria, diet-responsive B mitochondria, diet-resistant C, diet-responsive D, mitochondria from control mouse, *Ucp3*-knockout mitochondria, Cruz® MW markers. When possible, mitochondria from diet-responsive and diet-resistant subjects that were biopsied on the same day were run on the same gel.

2.4.3.6 RELATIVE-QUANTITATIVE REVERSE TRANSCRIPTION PCR (RQ-RT PCR)

Muscle biopsy specimens were thawed and homogenized in 1.0 ml of Tri-Reagent (Bio/Can, Mississauga, ON) at 10000 rpm for 1 min using a Virtashear homogenizer. The samples were stored at –20°C overnight, and RNA was isolated according to Tri-Reagent manufacturer's protocol. RNA concentration was determined spectrophotometrically using $OD_{260/280}$ and samples were stored at 4°C for further use. 2.5 ug of total RNA was used to synthesize 1st strand cDNA using 10 uM random decamer primers (Ambion, Austin, TX) and 200 Units of MMLV reverse transcriptase (Life Technologies, Burlington, ON) and incubating at 42°C for 1 hour. UCP3 primers (UCP3 forward 5'-CCTCGTTACCTTTCCACTGG-3' and UCP3 reverse 5'-GGCAGAGACAAAGTGGCAGG-3') were designed from human UCP3 cDNA sequence information to amplify a 615 bp sequence common to both known splice variants of the UCP3 mRNA. PCR was performed on 1ul of the RT reaction using 20 pmol of each primer and the Quantum RNA Classic 18s Internal Standards (Ambion, Austin, TX) following the manufacturer's protocol. Cocktails containing all components were used to reduce variation between samples. PCR products were run on 1% agarose gels and visualized with ethidium bromide staining. Band intensities were measured using the ChemiDoc apparatus and Quantity One software (Bio-Rad, Mississauga, ON). Relative intensity was

calculated by dividing the intensity of the 615 bp band corresponding to the UCP3 message by the 418 bp band corresponding to the 18s message. To compare ratios from different RT reactions, one sample was always used as an internal control, and therefore all relative mRNA levels were normalized to this internal control. These assays were done by André Gauthier, a graduate student in Dr. Ruth McPherson's laboratory. The results are presented exclusively in this thesis with André Gauthier's generous permission.

2.5 MATERIALS

Oligomycin, malonate, FCCP, BSA (fraction V), Nagarse (type XXVII), hexokinase (type III), succinic acid, GDP, ATP, ADP, rotenone, nigericin, succinate, stearic acid, linoleic acid, and lauric acid were purchased from Sigma®(St. Louis, MO). Tris-base, SDS, Tween-20, PMSF, β-mercaptoethanol, bromophenol, acrylamide/bisacrylamide and ammonium persulfate were purchased from Bio-Rad Laboratories (Hercules, CA). Radioactive compounds ($^{3}H_2O$, ^{14}C-sucrose, ^{14}C-methoxyinulin, $^{86}RbCl$, ^{36}Cl and ^{3}H-TPMP-Br) were purchased from Mandel Scientific Ltd and Dupont-NEN (Guelph, Ontario). All water insoluble compounds were dissolved in dimethyl sulfoxide (Sigma)

2.6 STATISTICAL ANALYSIS

Data were analyzed using two-tailed unpaired Student's t tests to determine differences between two individual means, or analyses of variance (One-way ANOVA and two-way ANOVA) when determining significance between more than two groups. Linear regression lines were compared by analysis of covariance. Statistics were performed using Prism® 3 for Windows. A p value of less than 0.05 was considered statistically significant. Unless otherwise stated, results are presented as means ± SEM.

3. RESULTS

3.1 UCP3-KNOCKOUT MICE

3.1.1 INCREASED BODY WEIGHT AND ADIPOSITY IN UCP3-KNOCKOUT MICE

Ucp3-knockout mice had a significantly higher body weight (40.9 g ± 1.61; n=8) than age-matched controls (32.0 ± 1.17; n=8)(p= 0.0005) (Figure 5). This difference can be attributed, at least in part, to significant increase in adiposity in the knockouts. Inguinal white adipose tissue (InWAT), interscapular BAT (IBAT), epididymal white adipose tissue (EWAT), and retroperitoneal white adipose tissue (RWAT) weights were significantly higher in *Ucp3*-knockout mice than controls (Figure 6).

3.1.2 COMPARISON OF THE KINETIC RESPONSES OF THE MITOCHONDRIAL PROTON LEAK IN SKELETAL MUSCLE MITOCHONDRIA FROM UCP3-KNOCKOUT AND WILD-TYPE CONTROL MICE

Results shown in Figure 7 are the product of a top-down elasticity analysis performed to assess the kinetic response of the proton leak in skeletal muscle mitochondria of knockouts and controls. These results show that at any give rate of oxygen consumption, mitochondrial protonmotive force is significantly higher in *Ucp3*-knockout mitochondria than in wild-type. Thus, there is a decreased proton leak in the skeletal muscle mitochondria of *Ucp3*-knockout over the complete range of metabolic rates studied. State 4 (maximal leak-dependent respiration) Δp values were significantly higher (p= 0.001) in *Ucp3*-knockout mitochondria than in control mitochondria (203.4 mV (±4.84) vs. 183.28 mV (±2.60); respectively). State 4 oxygen consumption values were not different at values of 127.35 nmol $O \cdot min^{-1} \cdot mg$

FIGURE 5. AVERAGE BODY WEIGHTS OF UCP3-KNOCKOUT AND WILD-TYPE CONTROL MICE FED A NORMAL CHOW DIET. Body weights were measured prior to sacrificing the animal for metabolic studies. Results are expressed as means. *** denotes a statistically significant difference (p=0.0005), as determined by Student's paired t-test

Body Weights

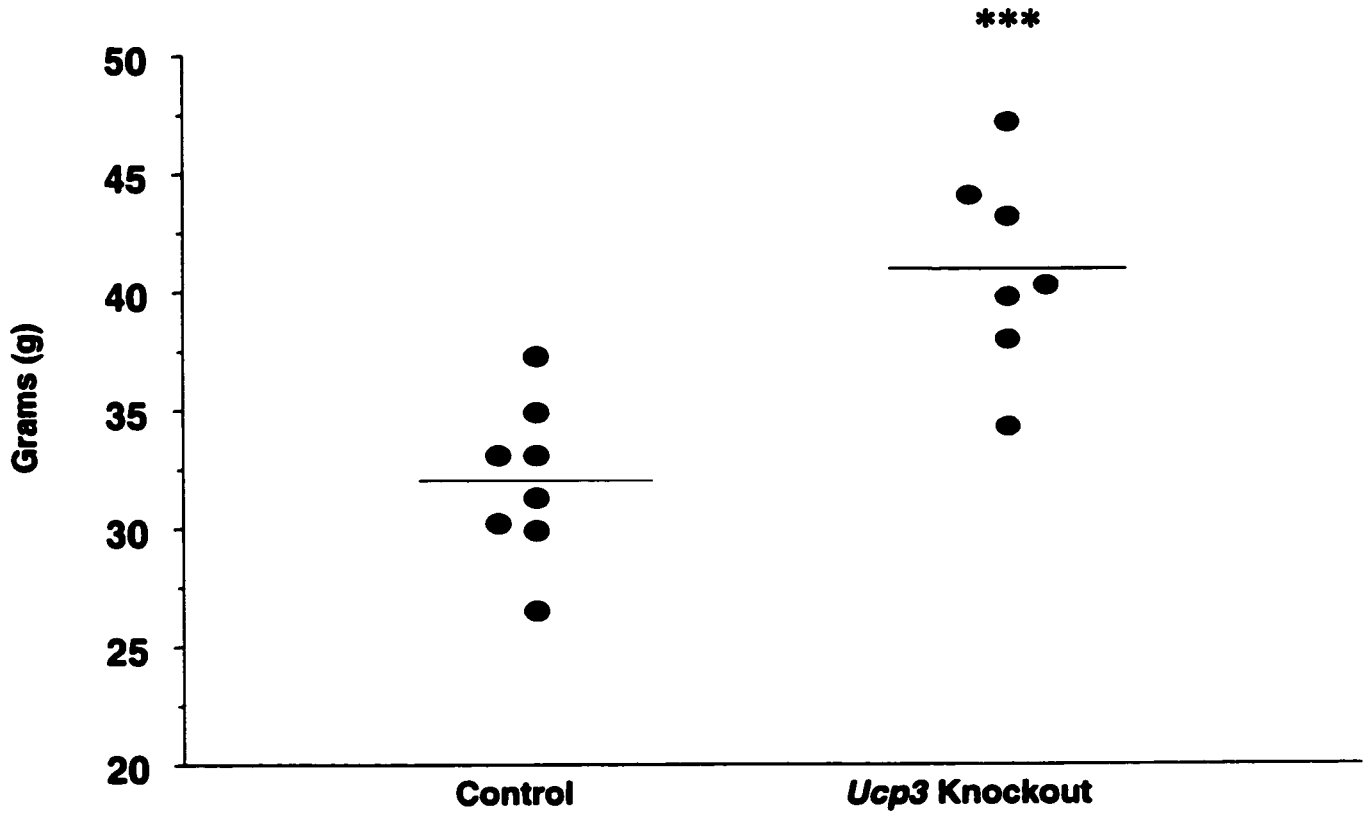


FIGURE 6. ADIPOSE TISSUE WEIGHTS OF UCP3-KNOCKOUT AND WILD-TYPE CONTROL MICE FED A NORMAL CHOW DIET. Tissues were removed immediately after the animal was sacrificed and kept on ice until they were weighed (no more than 30 min post-sacrifice). Results are expressed as means ±SEM. ** denotes a statistically significant difference between each of the paired columns at the level of $p<0.01$, *** at the level of $p<0.001$, as shown by ANOVA and Tukey's post-hoc tests.

Fat Pad Weights

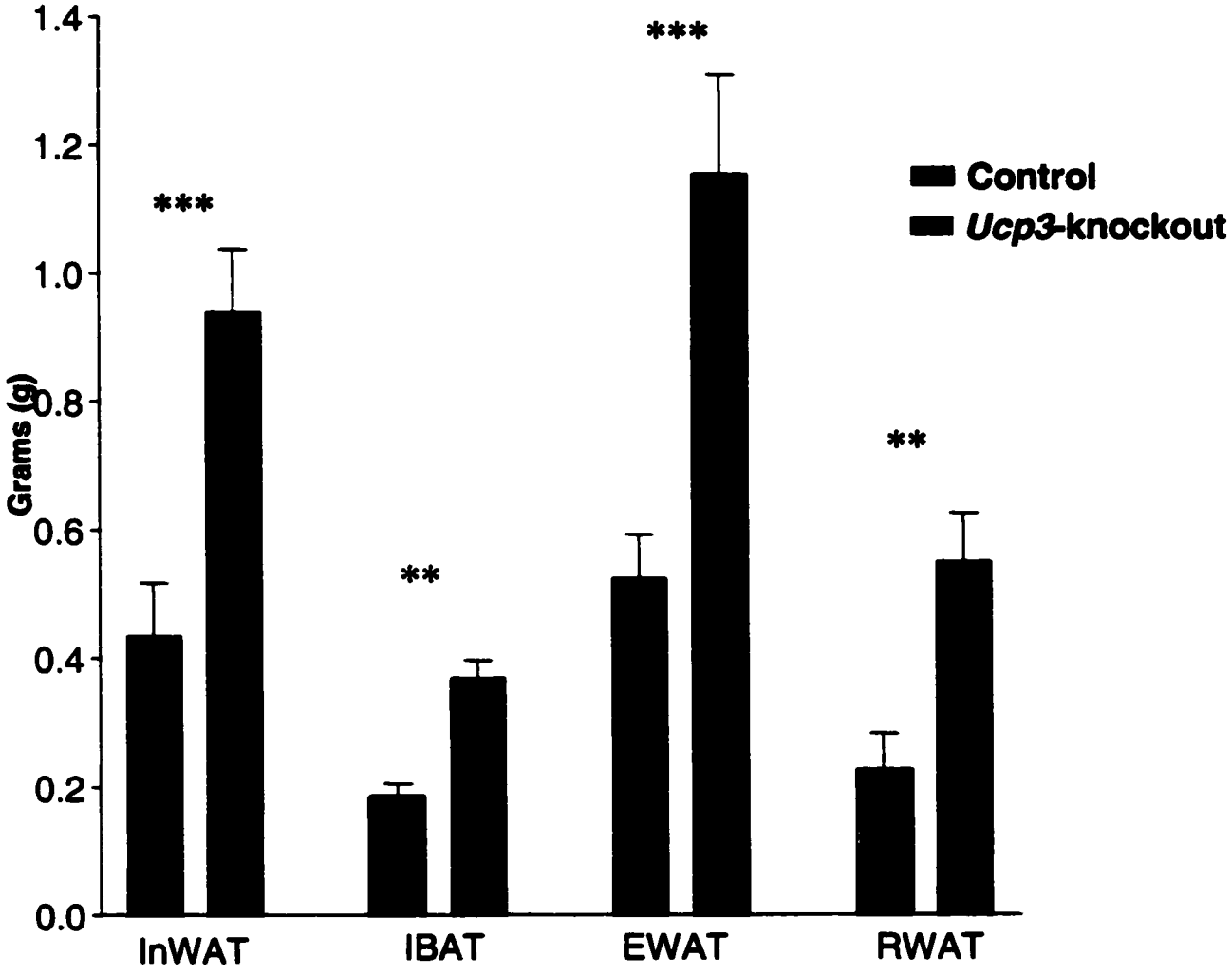
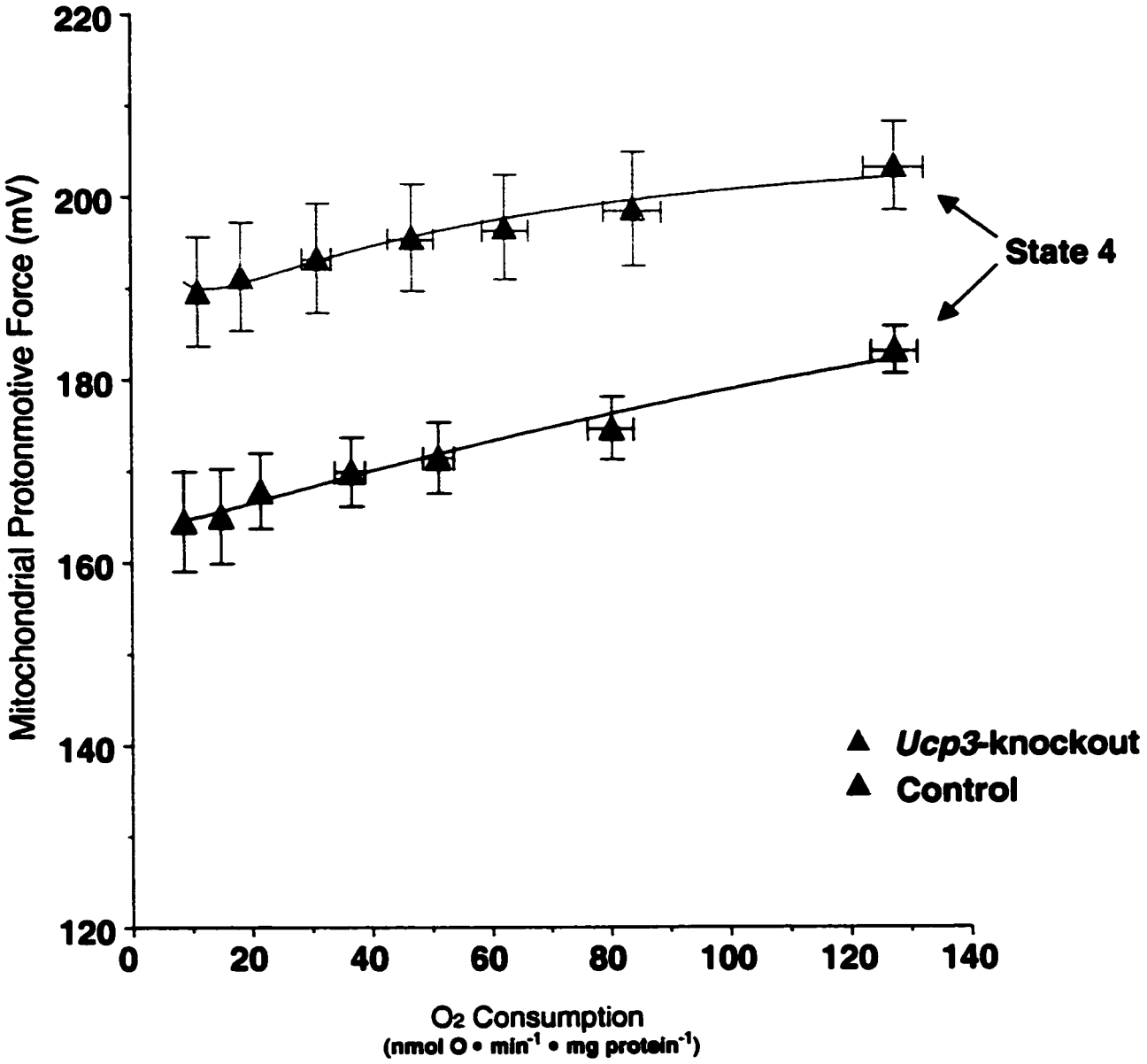


FIGURE 7. RELATIONSHIP BETWEEN ΔP AND LEAK-DEPENDENT RESPIRATION IN SKELETAL MUSCLE MITOCHONDRIA FROM UCP3-KNOCKOUT AND WILD-TYPE CONTROL MICE. The kinetic response of the proton leak to Δp was determined by titration of state 4 (maximal, non-phosphorylating) respiration with increasing amounts of a complex II inhibitor, malonate (0.33, 0.66, 1.0, 2.0, 3.0, and 5.0 mM) in the presence of saturating amounts of oligomycin (8 ug/mg mitochondrial protein). Each point represents the mean ±SEM of duplicate experiments with mitochondria from 8 mice.

Skeletal Muscle
Mitochondrial proton leak kinetic curves



protein^{-1} (±6.41) and 127.40 nmol O · min^{-1} · mg protein^{-1} (±5.62), for *Ucp3*-knockouts and controls, respectively.

3.1.3 Proton Leak in IBAT Mitochondria of UCP3-knockout and Control Mice

In IBAT mitochondria from *Ucp3*-knockout mice and controls, there was no significant difference in Δp or in the overall kinetics of the proton leak reactions, but there was a slightly lower state 4 respiration rate in knockout mitochondria than in controls (Figure 8). State 4 respiration values were 79.01 nmol O · min^{-1} · mg protein^{-1} (±3.51) and 65.44 nmol O · min^{-1} · mg protein^{-1} (±3.76) for wild-type mitochondria and knockout mitochondria, respectively ($p = 0.04$).

3.1.4 Proton Leak in Liver Mitochondria of UCP3-knockout and Control Mice

Since liver mitochondria do not contain UCP3, we studied mitochondria from this tissue as a negative control. As expected, liver mitochondria from *Ucp3*-knockout and control mice showed no detectable differences in state 4 oxygen consumption or in the overall characteristics of proton leak (Figure 9).

3.1.5 The Effect of Specific Free Fatty Acids on the Overall Kinetics of the Skeletal Muscle Mitochondrial Proton Leak in Mitochondria Lacking UCP3 and Wild-type Control Mitochondria

In the presence of 27 μM (f.c.) linoleic acid (18:2, n-6), we observed differences in the overall kinetics of the proton leak in wild-type mitochondria (Figure 10). In controls,

FIGURE 8. PROTON LEAK IN MITOCHONDRIA ISOLATED FROM THE IBAT OF UCP3-KNOCKOUT AND WILD-TYPE CONTROL MICE. The kinetic response of the proton leak to Δp was determined by titration of state 4 (maximal, non-phosphorylating) respiration with increasing amounts of a complex II inhibitor, malonate (0.33, 0.66, 1.0, 2.0, 3.0, and 5.0 mM) in the presence of saturating amounts of oligomycin (4 ug/mg mitochondrial protein). Each point represents the mean ±SEM of duplicate experiments with mitochondria from 6 mice.

INTERSCAPULAR BAT
MITOCHONDRIAL PROTON LEAK KINETIC CURVES

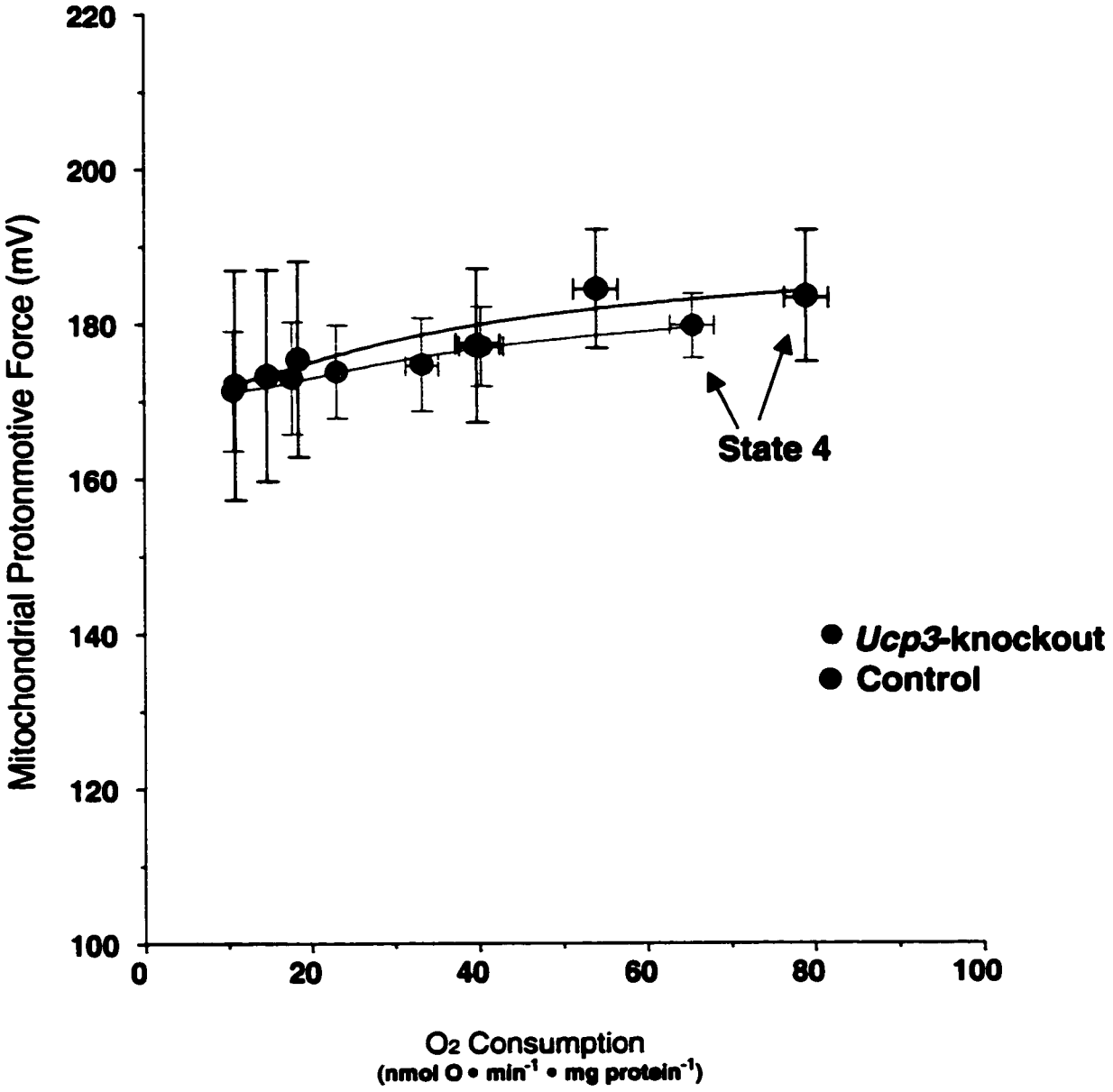
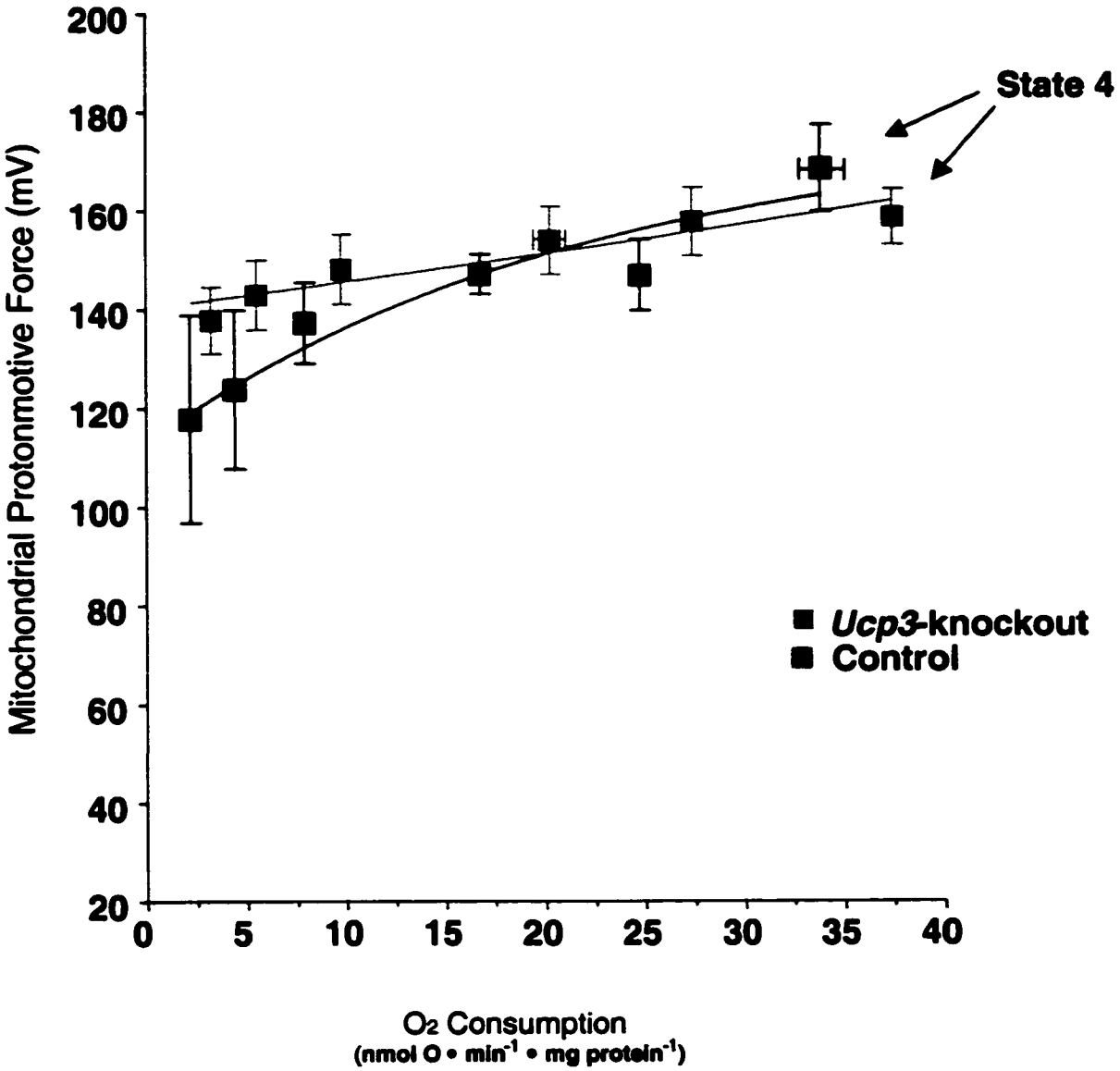


FIGURE 9. PROTON LEAK IN MITOCHONDRIA ISOLATED FROM LIVER OF UCP3-KNOCKOUT AND WILD-TYPE CONTROL MICE. The kinetic response of the proton leak to Δp was determined by titration of state 4 (maximal, non-phosphorylating) respiration with increasing amounts of a complex II inhibitor, malonate (0.33, 0.66, 1.0, 2.0, 3.0, and 5.0 mM) in the presence of saturating amounts of oligomycin (8 ug/mg mitochondrial protein). Each point represents the mean ±SEM of duplicate measurements with mitochondria from 3 mice.

LIVER
MITOCHONDRIAL PROTON LEAK KINETIC CURVES

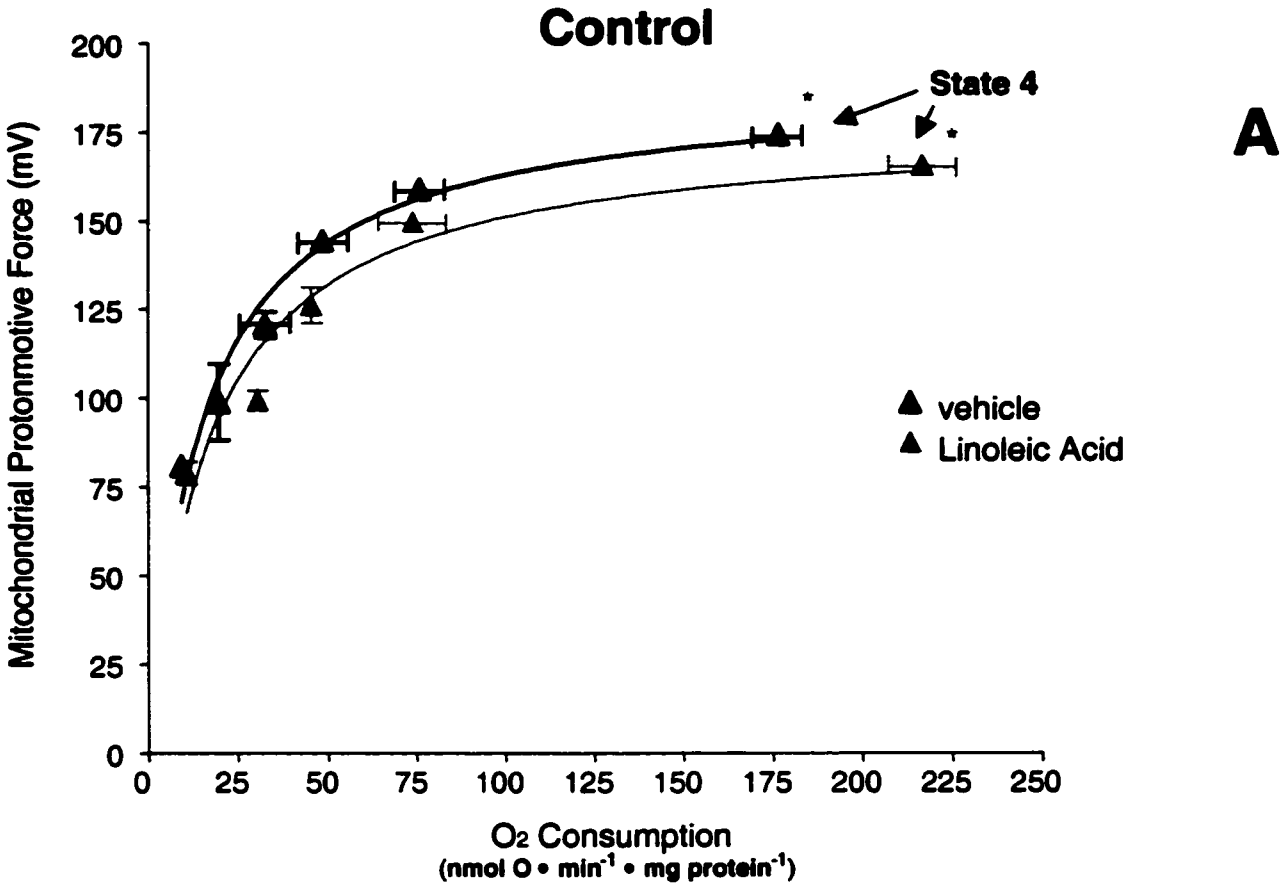


State 4 oxygen consumption significantly increased from 176.53 nmol O · min^{-1} · mg $protein^{-1}$ (± 1.49) to 216.54 nmol O · min^{-1} · mg $protein^{-1}$ (± 1.54) (p= 0.005) in the presence of linoleate (Figure 10A). State 4 protonmotive force dropped from 174.75 mV (± 1.54) to 165 mV (± 1.49) (p=0.011; Figure 10A). Incubating *Ucp3*-knockout mitochondria with linoleate had no significant effects on state 4 values for oxygen consumption and protonmotive force, or on the overall kinetics of the leak (Figure 10B).

Incubation of wild-type mitochondria with 66 μM of lauric acid (12:0) produced similar results as those obtained with the addition of linoleic acid. State 4 oxygen consumption was significantly higher in the presence of laurate (228.43 nmol O · min^{-1} · mg $protein^{-1}$ ± 18.4) than in its absence (176.53 nmol O · min^{-1} · mg $protein^{-1}$ ± 1.49), and state 4 Δp significantly dropped from 174.75 mV (± 1.54) to 161.21 mV (± 7.12) in the wild-type mitochondria (Figure 11A). The addition of lauric acid to *Ucp3*-knockout mitochondria had no impact on the kinetics of the proton leak or on state 4 values (Figure 11B).

Finally, we looked at the effects of adding 27 μM stearic acid (18:0) to our mitochondrial incubations. Stearic acid, like the other free fatty acids used, had no effect on the kinetic responsiveness of the proton leak or on state 4 values when added to *Ucp3*-knockout mitochondria (Figure 12B). Stearate did, however, significantly increase mitochondrial oxygen consumption in wild-type mitochondria from 176.53 nmol O · min^{-1} · mg $protein^{-1}$ (± 1.49) to 222.21 nmol O · min^{-1} · mg $protein^{-1}$ (± 16.5) (p= 0.008; Figure 12A). The addition of stearate did not significantly affect Δp values in the controls.

FIGURE 10. THE EFFECT OF LINOLEIC ACID ON PROTON LEAK AND OXYGEN CONSUMPTION IN SKELETAL MUSCLE MITOCHONDRIA OF WILD-TYPE CONTROL (A) AND UCP3-KNOCKOUT (B) MICE. The kinetic response of the proton leak to Δp was determined by titration of state 4 (maximal, non-phosphorylating) respiration with saturating amounts of oligomycin (8 ug/mg mitochondrial protein) and increasing amounts of a complex II inhibitor, malonate (0.33, 0.66, 1.0, and 2.0 mM) in the presence of 27 μM linoleic acid. Each point represents the mean ±SEM of duplicate experiments with mitochondria from 7 mice. * denotes a significant effect of linoleic acid.


Control
State 4
A
vehicle
Linoleic Acid
Mitochondrial Protonmotive Force (mV)
O2 Consumption
(nmol O • min-1 • mg protein-1)
0
25
50
75
100
125
150
175
200
225
250

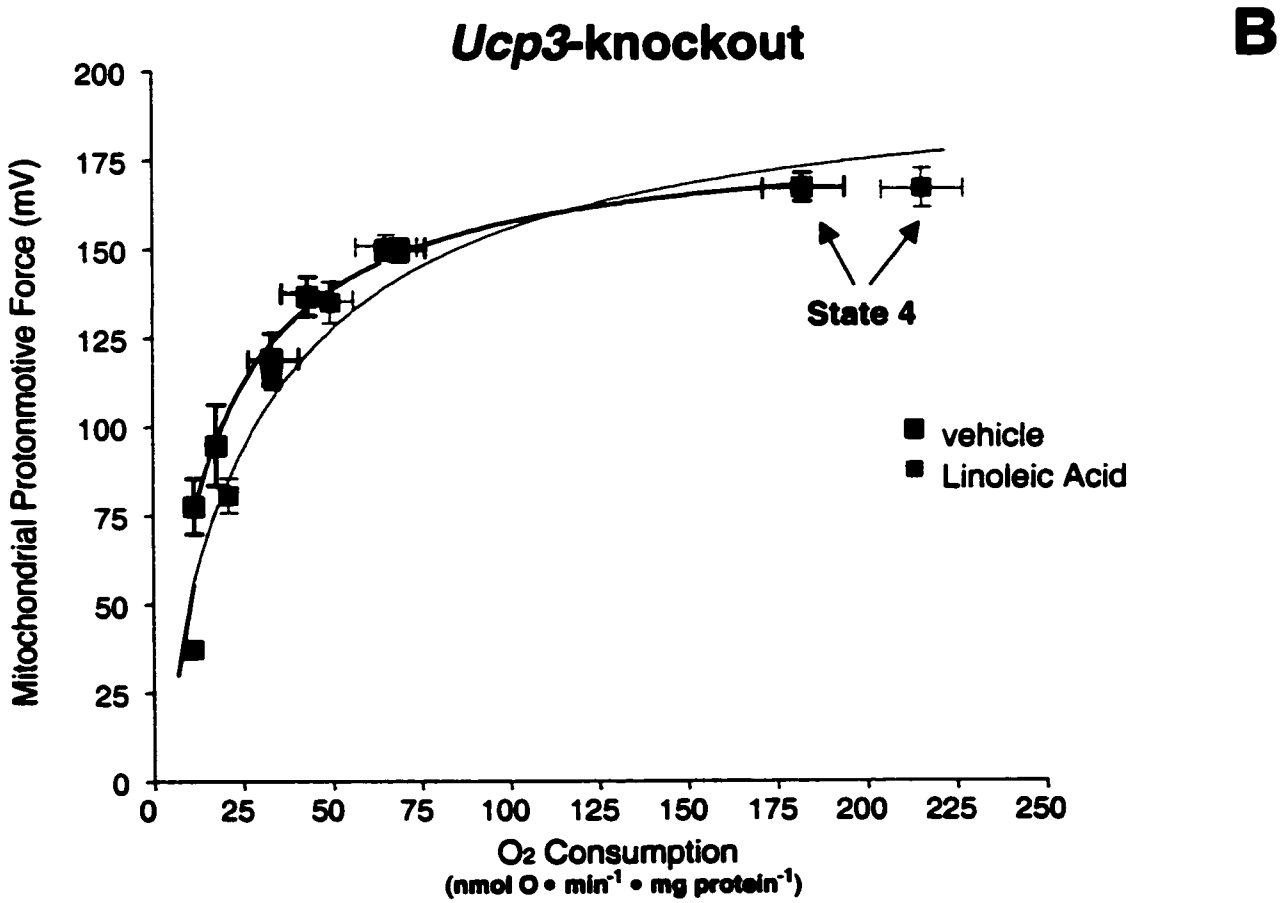

Ucp3-knockout
B
State 4
vehicle
Linoleic Acid
Mitochondrial Protonmotive Force (mV)
O2 Consumption
(nmol O • min-1 • mg protein-1)
0
25
50
75
100
125
150
175
200
225
250

FIGURE 11. THE EFFECT OF LAURIC ACID ON PROTON LEAK AND OXYGEN CONSUMPTION IN SKELETAL MUSCLE MITOCHONDRIA OF WILD-TYPE CONTROL (A) AND UCP3-KNOCKOUT (B) MICE. The kinetic response of the proton leak to Δp was determined by titration of state 4 (maximal, non-phosphorylating) respiration with saturating amounts of oligomycin (8 ug/mg mitochondrial protein) and increasing amounts of a complex II inhibitor, malonate (0.33, 0.66, 1.0, and 2.0 mM) in the presence of 66 μM lauric acid. Each point represents the mean ±SEM of duplicate experiments with mitochondria from 7 mice. * denotes a significant effect of lauric acid.

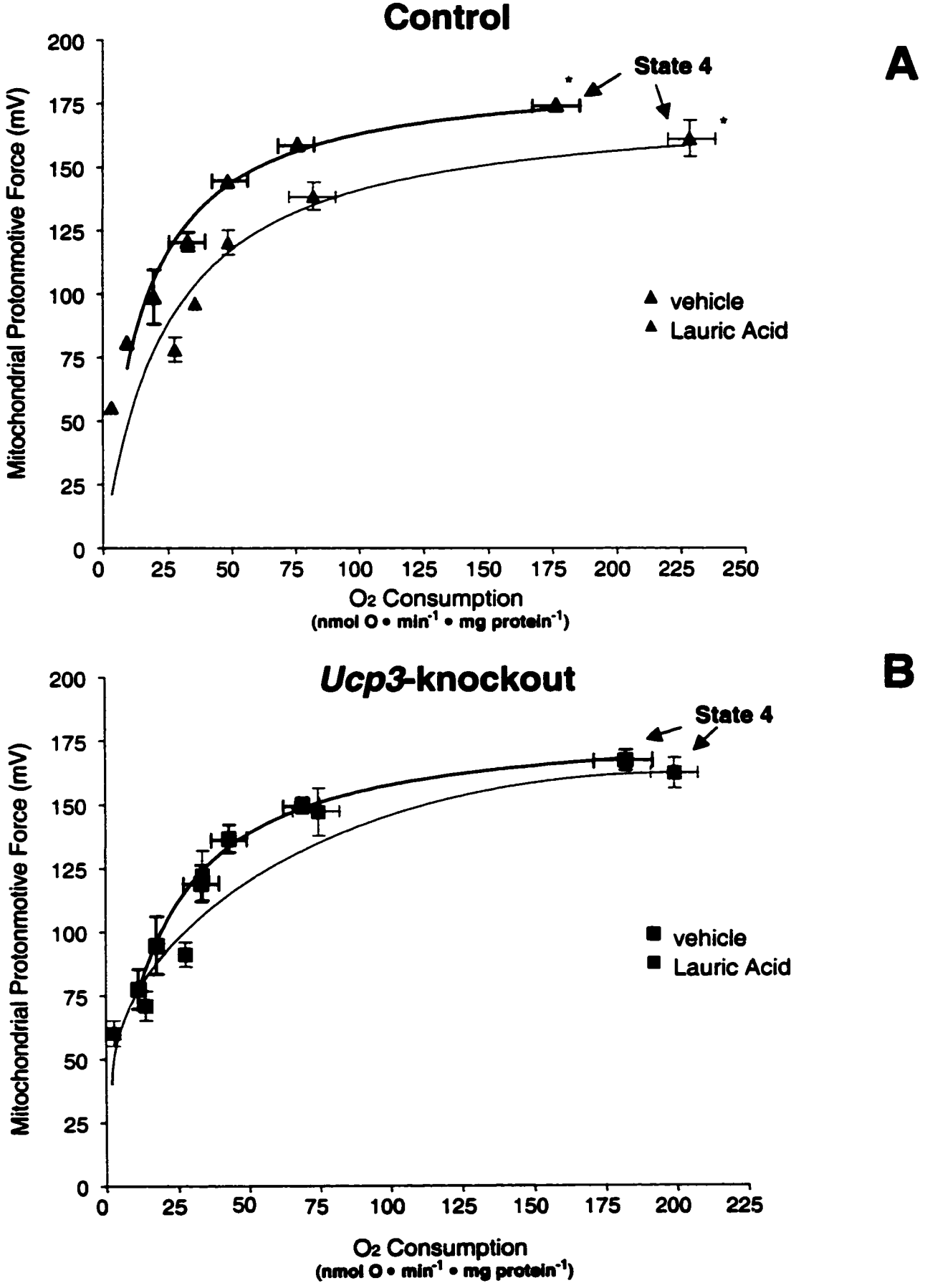
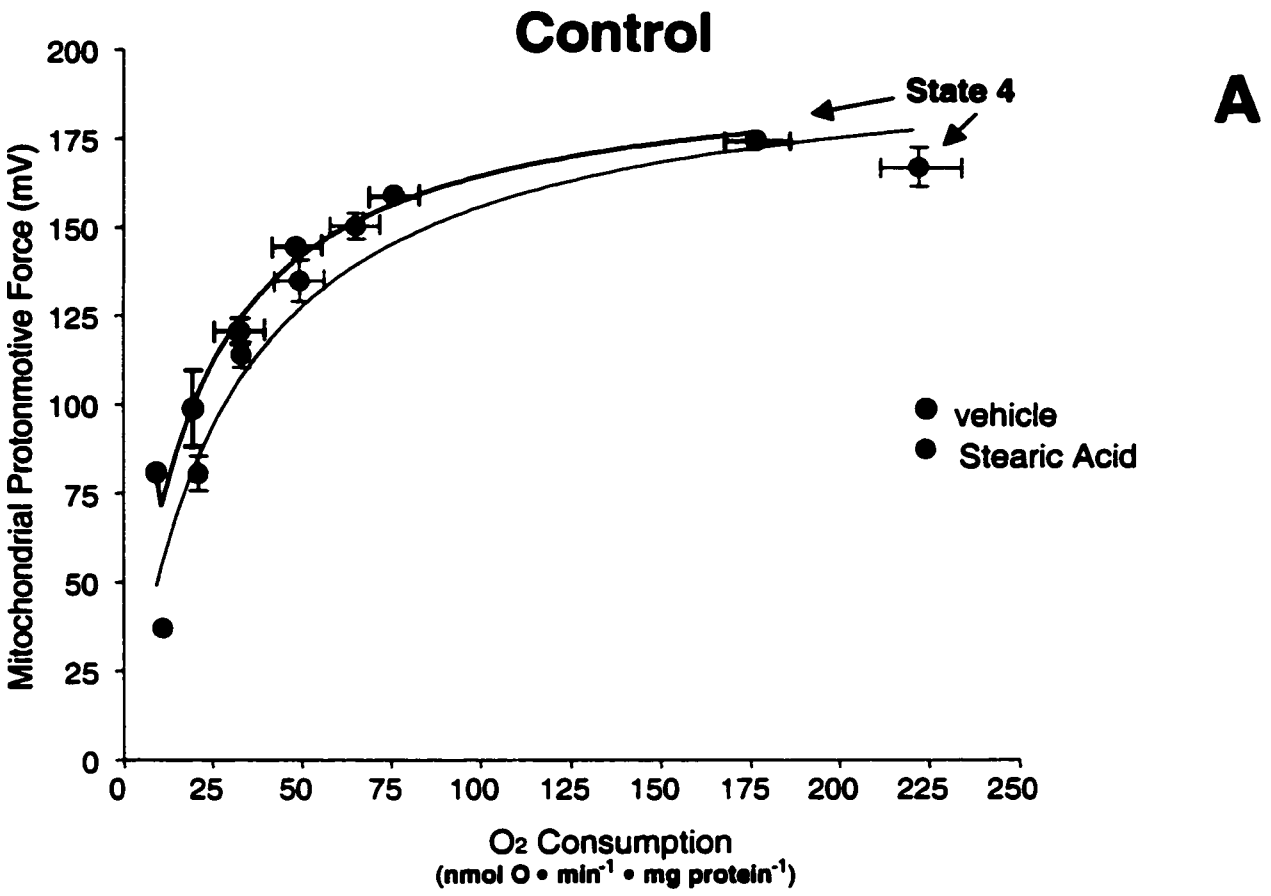
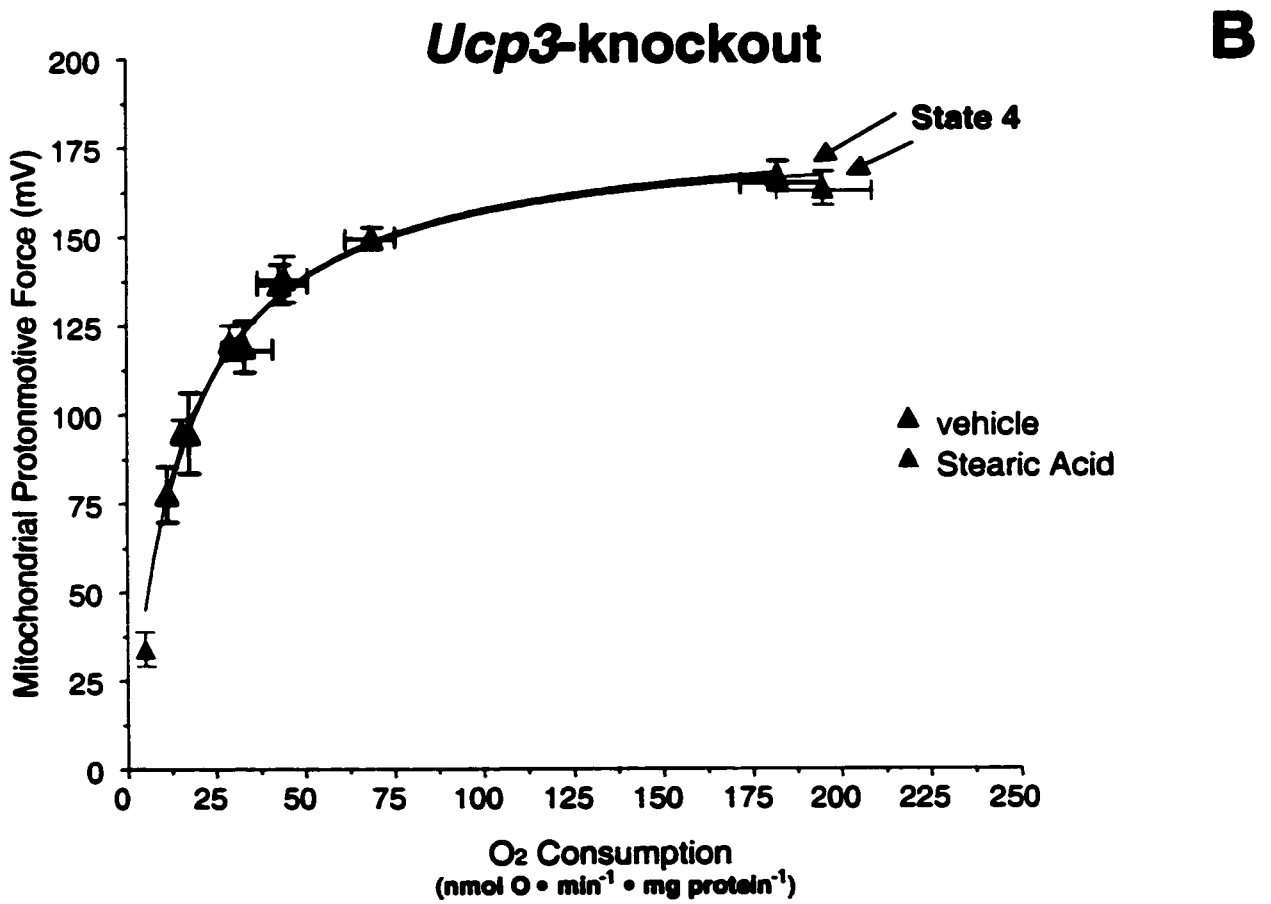

Control
A
State 4
Mitochondrial Protonmotive Force (mV)
200
175
150
125
100
75
50
25
0
0
25
50
75
100
125
150
175
200
225
250
O2 Consumption
(nmol O • min-1 • mg protein-1)
vehicle
Lauric Acid
Ucp3-knockout
B
State 4
Mitochondrial Protonmotive Force (mV)
200
175
150
125
100
75
50
25
0
0
25
50
75
100
125
150
175
200
225
O2 Consumption
(nmol O • min-1 • mg protein-1)
vehicle
Lauric Acid

FIGURE 12. THE EFFECT OF STEARIC ACID ON PROTON LEAK AND OXYGEN CONSUMPTION IN SKELETAL MUSCLE MITOCHONDRIA OF WILD-TYPE CONTROL (A) AND UCP3-KNOCKOUT (B) MICE. The kinetic response of the proton leak to Δp was determined by titration of state 4 (maximal, non-phosphorylating) respiration with saturating amounts of oligomycin (8 ug/mg mitochondrial protein) and increasing amounts of a complex II inhibitor, malonate (0.33, 0.66, 1.0, and 2.0 mM) in the presence of 27 μM stearic acid. Each point represents the mean ±SEM of duplicate experiments with mitochondria from 7 mice.


Control
A
State 4
vehicle
Stearic Acid
Mitochondrial Protonmotive Force (mV)
O2 Consumption
(nmol O • min-1 • mg protein-1)
0
25
50
75
100
125
150
175
200
225
250


Ucp3-knockout
B
State 4
vehicle
Stearic Acid
Mitochondrial Protonmotive Force (mV)
O2 Consumption
(nmol O • min-1 • mg protein-1)
0
25
50
75
100
125
150
175
200
225
250

3.2 Mice Overexpressing Ucp3 (UCP-3Tg)

3.2.1 Body Weight and Tissue Analysis

As shown in Figure 13, the body weights of mice overexpressing Ucp3 were not significantly different from controls. The values were 27.98g ± 0.84 and 29.68g 0.56 for UCP-3Tg and wild-type controls, respectively (p=0.11).

For our tissue analyses we removed InWAT, IBAT, EWAT, and the heart. As shown in Figure 14, control mice had a greater InWAT wet weight (0.55g ±0.07) than UCP-3Tg (0.39g ±0.04) ($p<0.05$, n=17; one-way ANOVA). IBAT weights (shown in Figure 14) were found to be similar between both groups of mice (UCP-3Tg: 0.13g (±0.006); controls: 0.13g (±0.02); $p>0.05$, n=11). EWAT depots weights were also not significantly different between UCP-3Tg (0.70g ±0.08, n=17) and controls (0.55g ±0.11, n=17). Heart weights were comparable between the two groups (UCP-3Tg: 0.14g ±0.004; controls: 0.14g ±0.007; n=17).

3.2.2 Mitochondrial Proton Leak Assessments

The results in Figure 15 clearly show that the overall kinetics of the proton leak is significantly altered in mice overexpressing Ucp3 as compared to controls. State 4 oxygen consumption of UCP-3Tg mitochondria (177.42 nmol O · min^{-1} · mg $protein^{-1}$ ±6.3) was significantly higher (p=0.048) as compared to control values (155.97 nmol O · min^{-1} · mg $protein^{-1}$ ±8.4). In addition, for any given value of Δp, there is a higher amount of oxygen which needs to be consumed in order to support the proton leak reactions. Conversely, state 4 mitochondrial protonmotive force values were lower in the UCP-3Tg mice compared to

FIGURE 13. AVERAGE BODY WEIGHTS OF MICE OVEREXPRESSING UCP3 (UCP-3TG) AND WILD-TYPE CONTROL MICE FED A NORMAL CHOW DIET. Body weights were measured prior to sacrificing the animal for metabolic studies. Results are expressed as means calculated from 17 UCP-3Tg and 15 control mice. Means were not statistically different between the two groups of mice ($p=0.11$; as determined by Student's paired t-test).

Body Weights

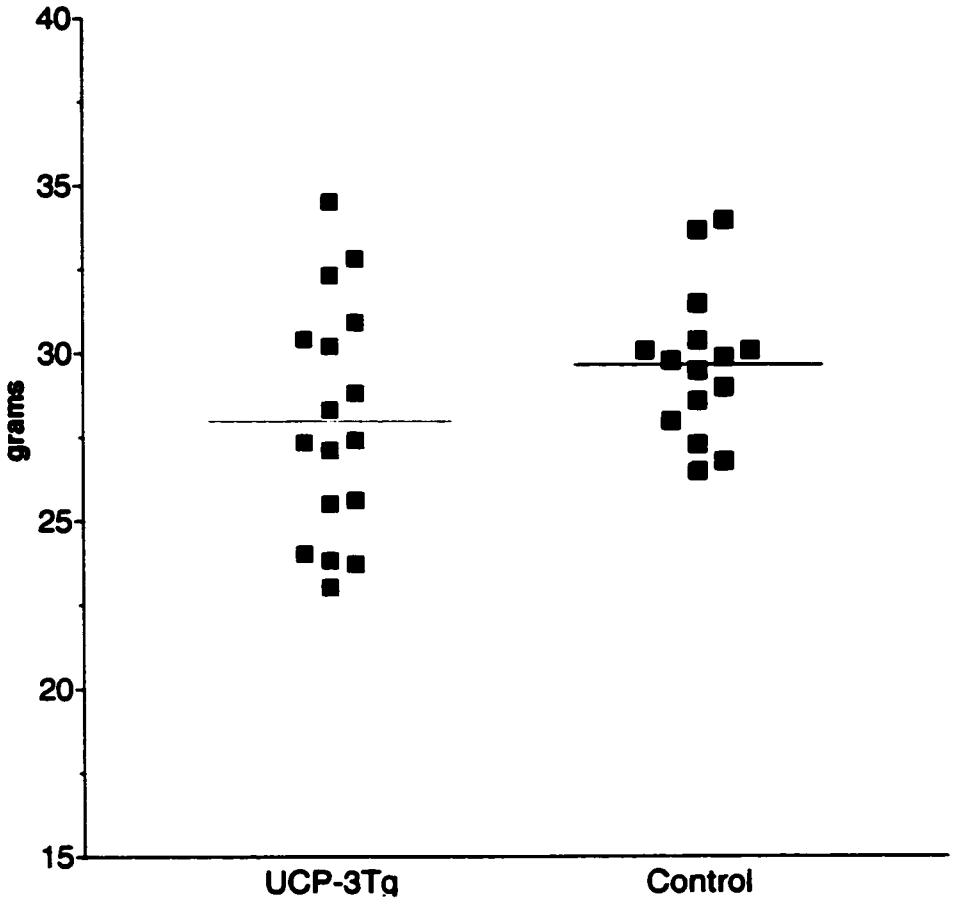


FIGURE 14. TISSUE WEIGHTS OF UCP-3TG AND WILD-TYPE CONTROL MICE ON A NORMAL CHOW DIET. Tissues were removed immediately after the animal was sacrificed and kept on ice until they were weighed (no more than 30 min post-sacrifice). Results are expressed as means for 12 mice in each group. There was no statistically significant difference between the means of the paired columns in any fat depot except for InWAT (* denotes $p<0.05$, as determined by a one-way ANOVA and Tukey's post hoc tests).

Tissue Weights

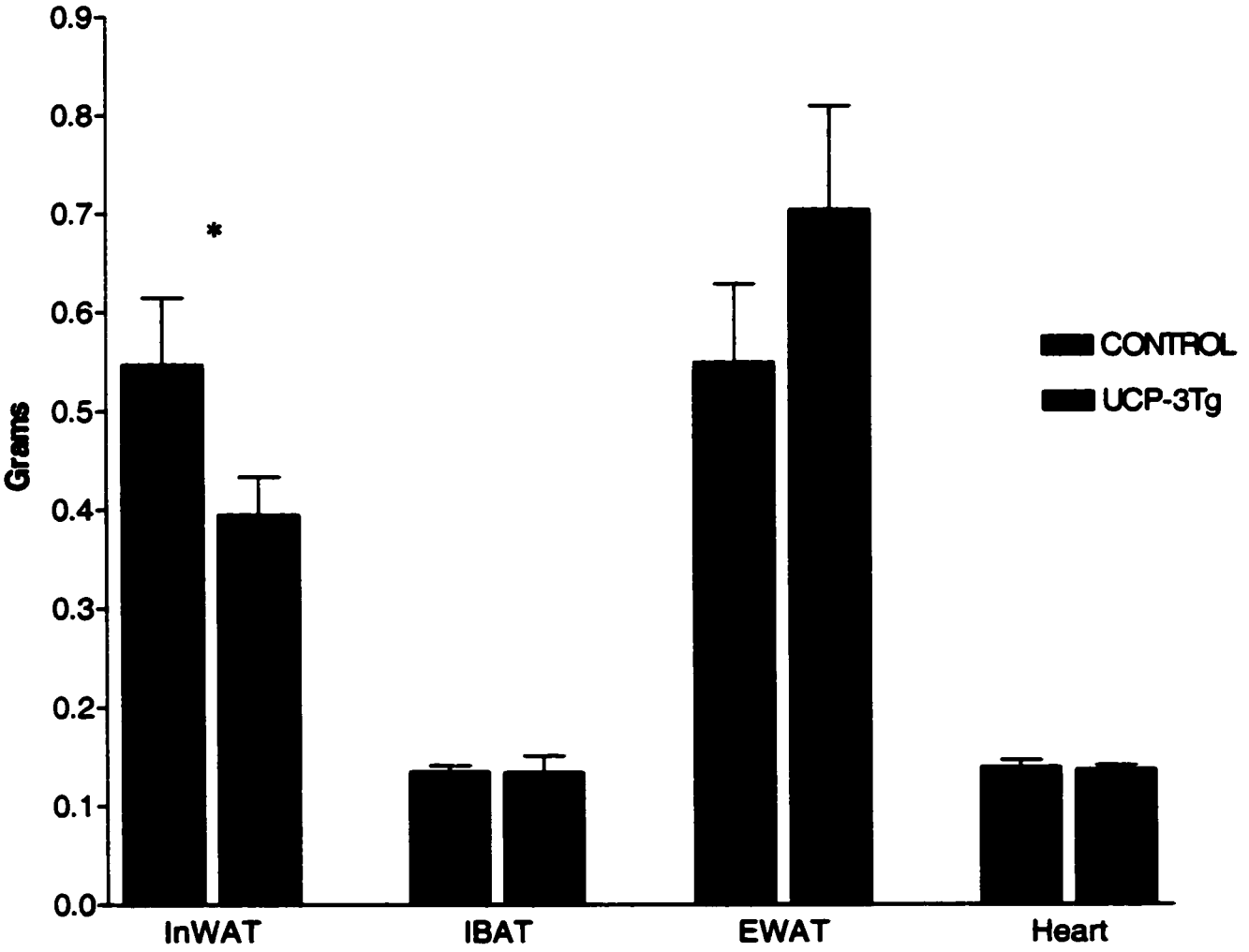
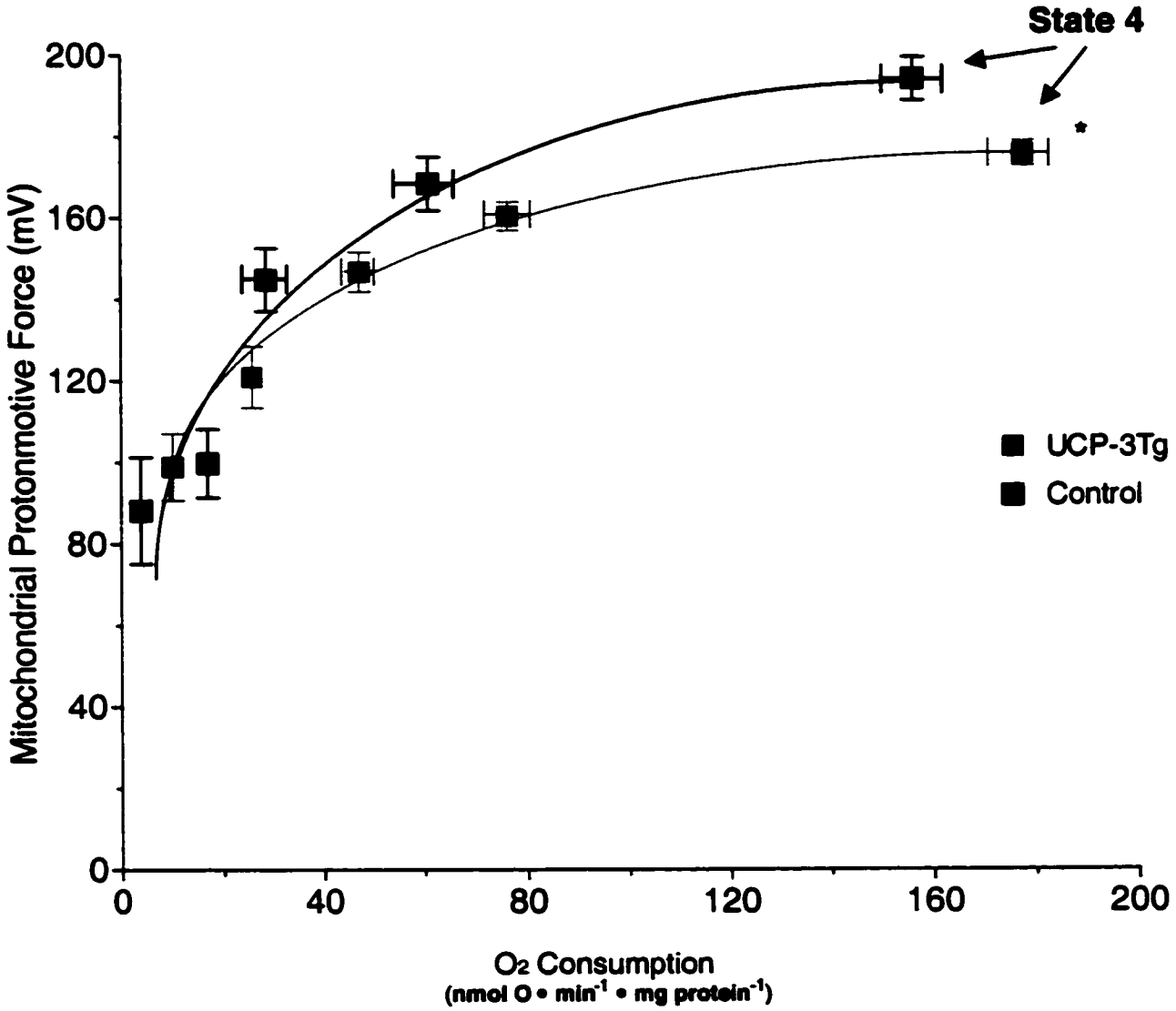


FIGURE 15. RELATIONSHIP BETWEEN Δp AND LEAK-DEPENDENT RESPIRATION IN SKELETAL MUSCLE MITOCHONDRIA FROM MICE OVEREXPRESSING UCP3 AND WILD TYPE CONTROL MICE. The kinetic response of the proton leak to Δp was determined by titration of state 4 (maximal, non-phosphorylating) respiration with increasing amounts of a complex II inhibitor, malonate (0.66, 1.0, 2.0, and 5.0 mM) in the presence of saturating amounts of oligomycin (8 ug/mg mitochondrial protein). Each point represents the mean ±SEM of duplicate experiments with mitochondria from 8 mice. * State 4 *oxygen consumption* is significantly greater ($p= 0.048$) and proton motive force significantly lower ($p= 0.008$) in mitochondria from UCP3-Tg mice than in those from wild-type control mice (unpaired Student's t-test).

Skeletal Muscle
Mitochondrial proton leak kinetic curves



controls (UCP-3Tg: 176.22 mV ±3.1; controls: 194.70 mV±5.3; p=0.008). So, for any given metabolic rate (oxygen consumption rate), Δp is lower in UCP-3Tg mitochondria compared to controls. In other words, the rate of mitochondrial proton leak is higher in the UCP3 overexpressors.

3.2.4 Western Blotting

Western blotting analyses show that mitochondria isolated from skeletal muscle of UCP-3Tg mice contain more UCP3 than those isolated from wild-type controls (Figure 16).

3.2.5 Histological Studies of Adipose and Muscle Tissues

Figures 17-22 show histology of EWAT, InWAT, IBAT, quadricep muscle, soleus muscle, and gastrocnemius muscle, respectively. Analyses of adipose tissue sections revealed that adipocytes are smaller in tissues taken from UCP-3Tg mice compared to controls. There was no visible difference in the appearance of any of the muscle tissue sections studied from either mouse group.

FIGURE 16. A REPRESENTATIVE WESTERN BLOTS OF SKELETAL MUSCLE MITOCHONDRIAL PREPARATIONS FROM UCP-3TG AND CONTROL MICE PROBED USING AN ANTIBODY AGAINST PURIFIED RABBIT UCP3. The location and size of biotinylated molecular weight markers (lane 1) correspond to those of Rainbow™ molecular weight markers depicted on the left of the blot. Lanes 3 and 5 both contain skeletal muscle mitochondria from wild-type control mice, whereas lanes 4 and 6 contain skeletal muscle mitochondria from UCP-3Tg mice. Lane 7 contains skeletal muscle mitochondria from *Ucp3*-knockout mice. The primary antibody used was a polyclonal rabbit anti-human UCP3; the secondary antibody was an anti-rabbit IgG horseradish peroxidase conjugate. Exposure time was 5 minutes.

Western Blots

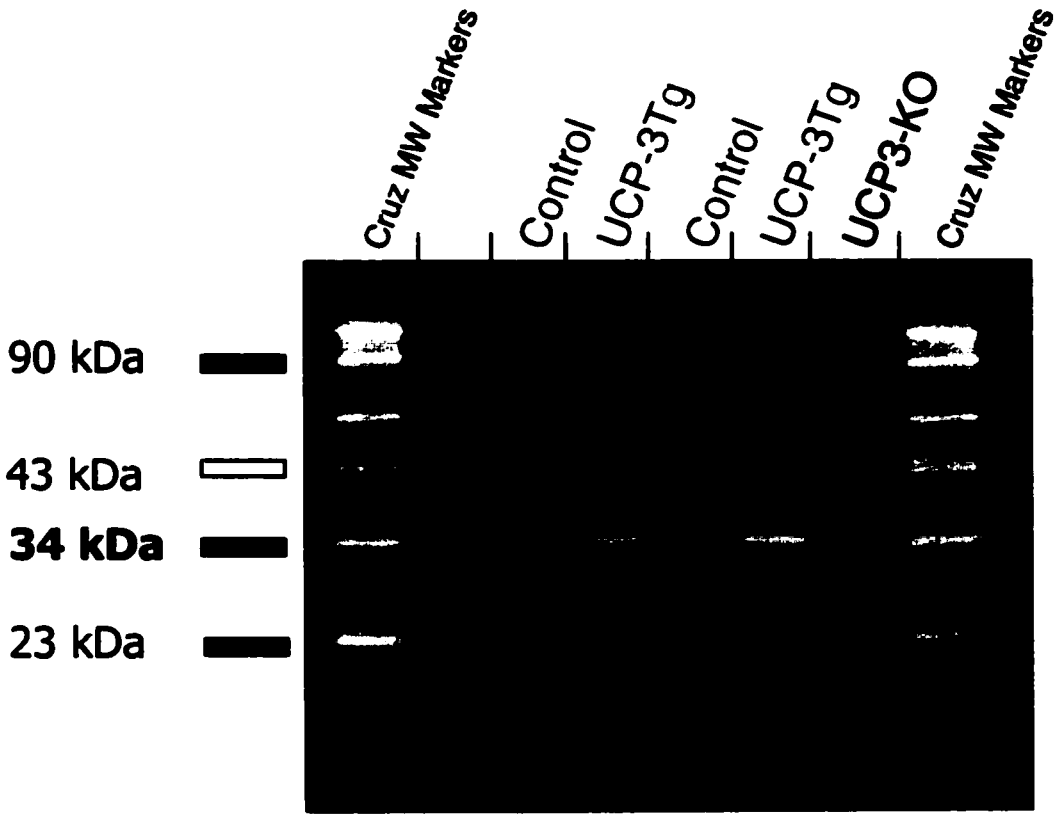
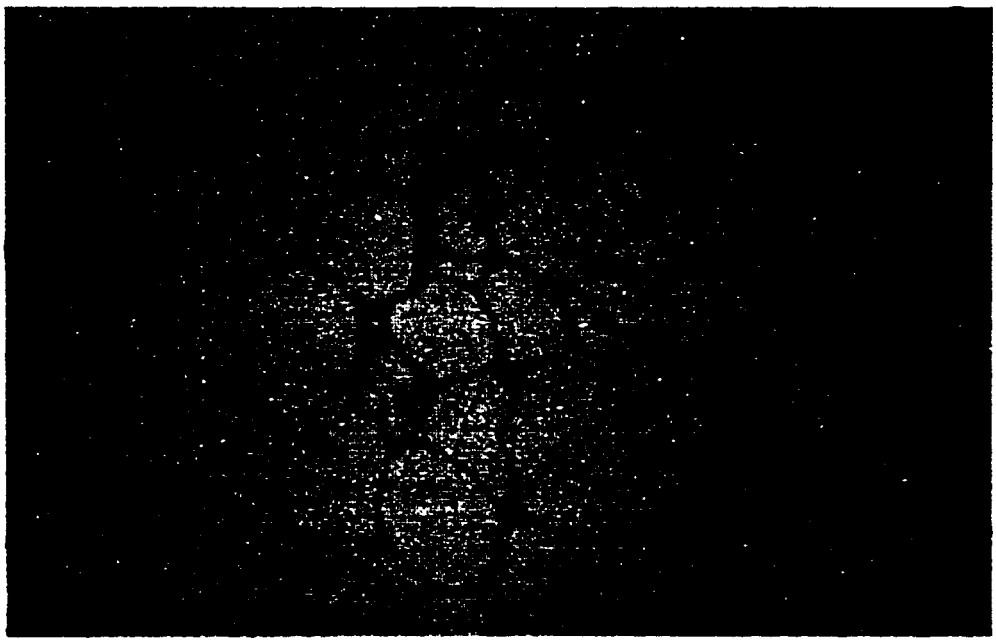


FIGURE 17. HISTOLOGY OF EPIDIDYMAL WHITE ADIPOSE TISSUE (EWAT). Hematoxylin and eosin stained 4μm sections of EWAT from UCP-3Tg (top) and control (bottom) mice fed a normal chow diet. Magnification x 400. Note the decreased size of lipid droplets in the unilocular cells of the tissue from the transgenic mouse as compared to those from the control.

Epididymal WAT Histology

UCP-3Tg



Control

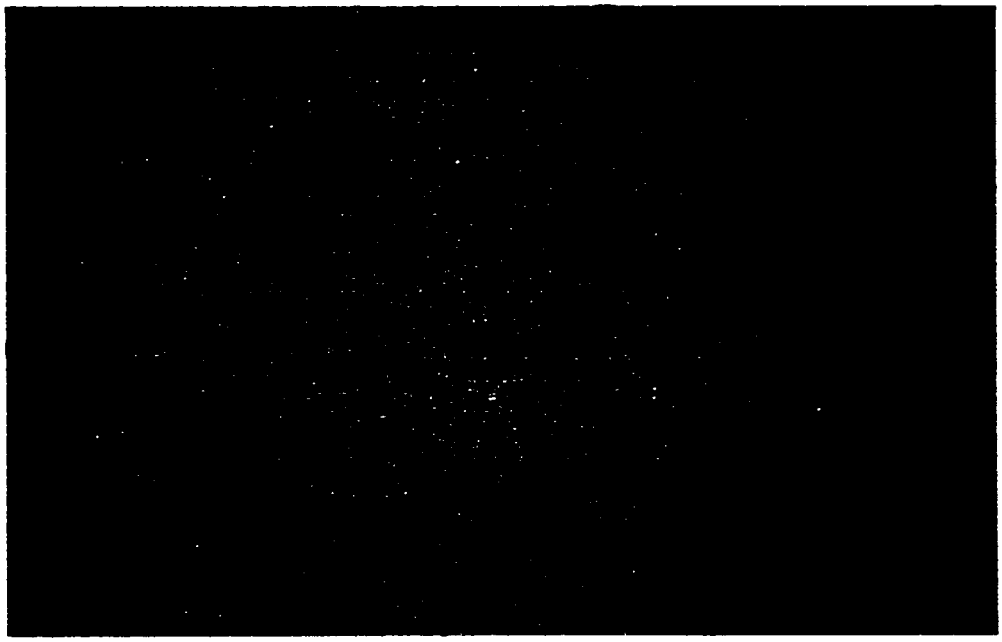
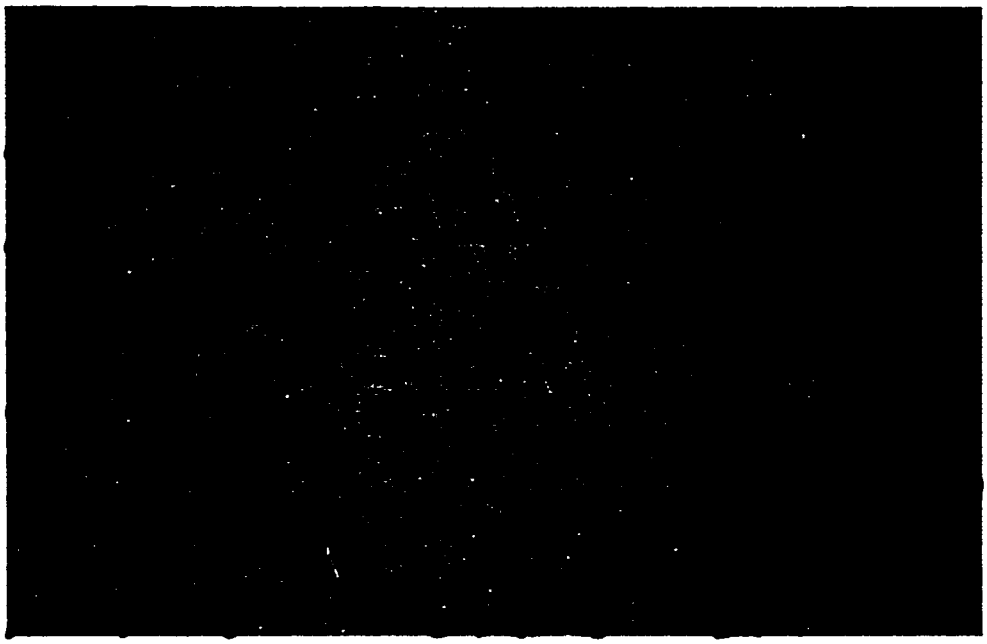


FIGURE 18. HISTOLOGY OF INGUINAL WHITE ADIPOSE TISSUE (INWAT). Hematoxylin and eosin stained 4μm sections of INWAT from UCP-3Tg (top) and control (bottom) mice fed a normal chow diet. Magnification x 400. Note smaller diameter of cells from UCP-3Tg mice than of cells from control mice. Note smaller diameter of cells from UCP-3Tg mice than of cells from control mice.

Inguinal WAT Histology

UCP-3Tg



Control

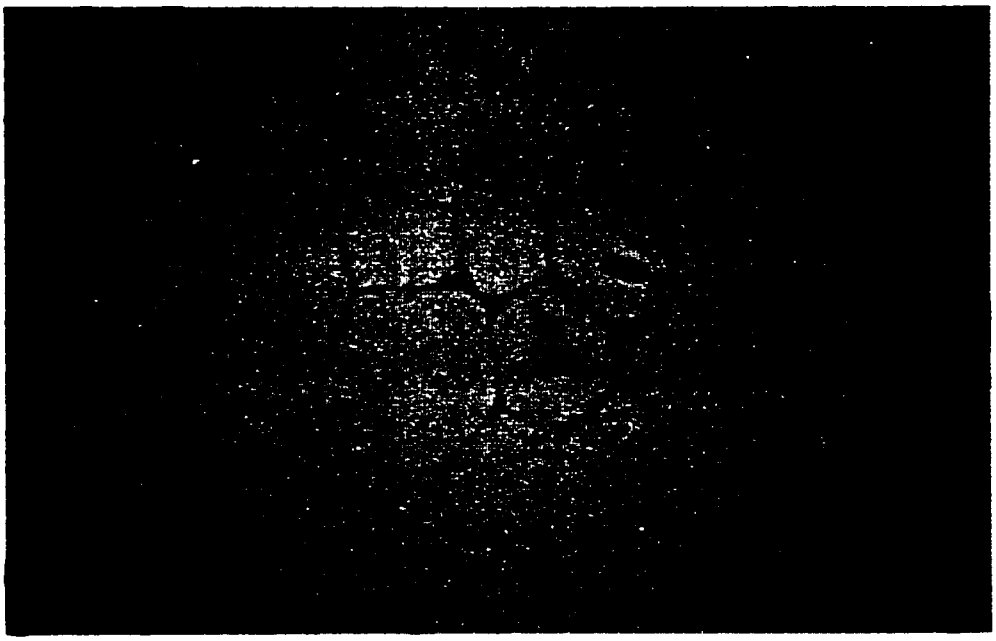
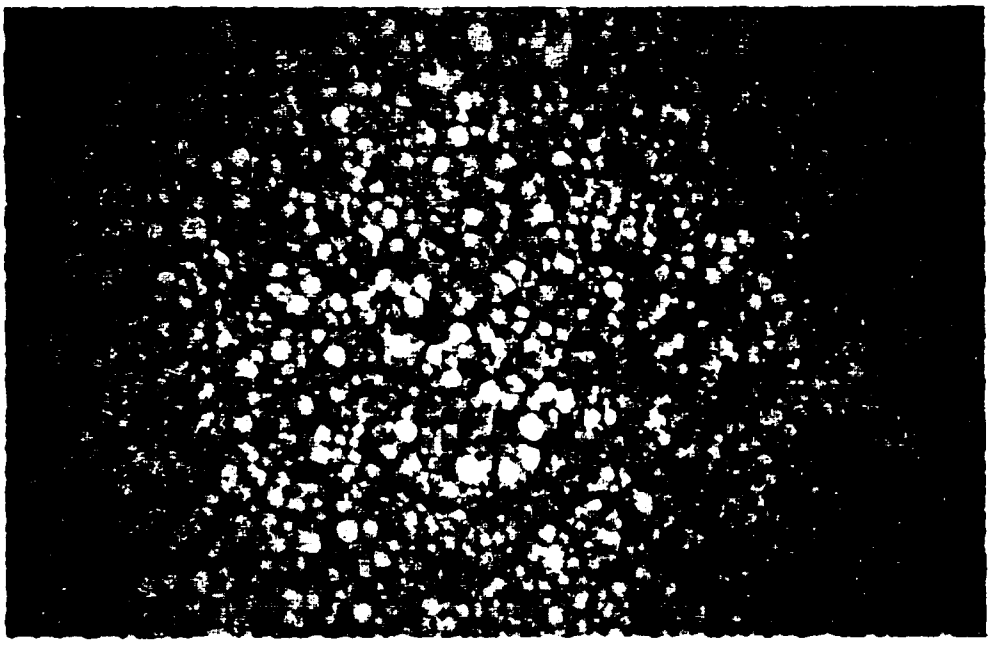


FIGURE 19. HISTOLOGY OF INTERSCAPULAR BROWN ADIPOSE TISSUE (IBAT). Hematoxylin and eosin stained 4μm sections of BAT from UCP-3Tg (top) and control (bottom) mice fed a normal chow diet. Magnification x 400. Note smaller diameter of cells from UCP-3Tg mice than of cells from control mice.

BAT Histology

UCP-3Tg



Control

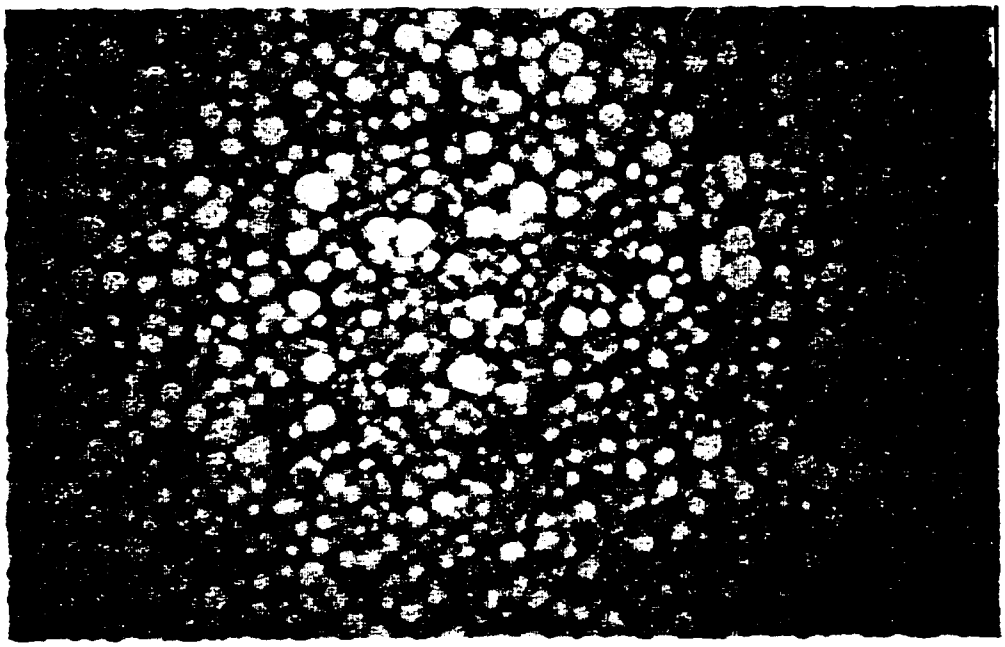
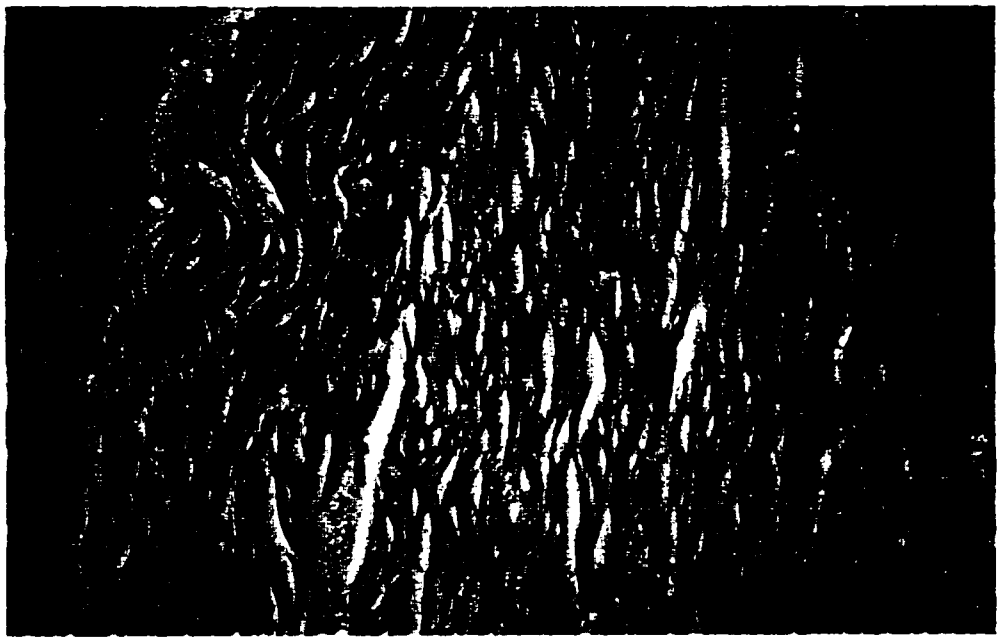


FIGURE 20. HISTOLOGY OF QUADRICEP MUSCLE. Hematoxylin and eosin stained 4μm sections of quadricep muscle from UCP-3Tg (top) and control (bottom) mice fed a normal chow diet. Magnification x 400.

Quadricep Muscle Histology

UCP-3Tg



Control

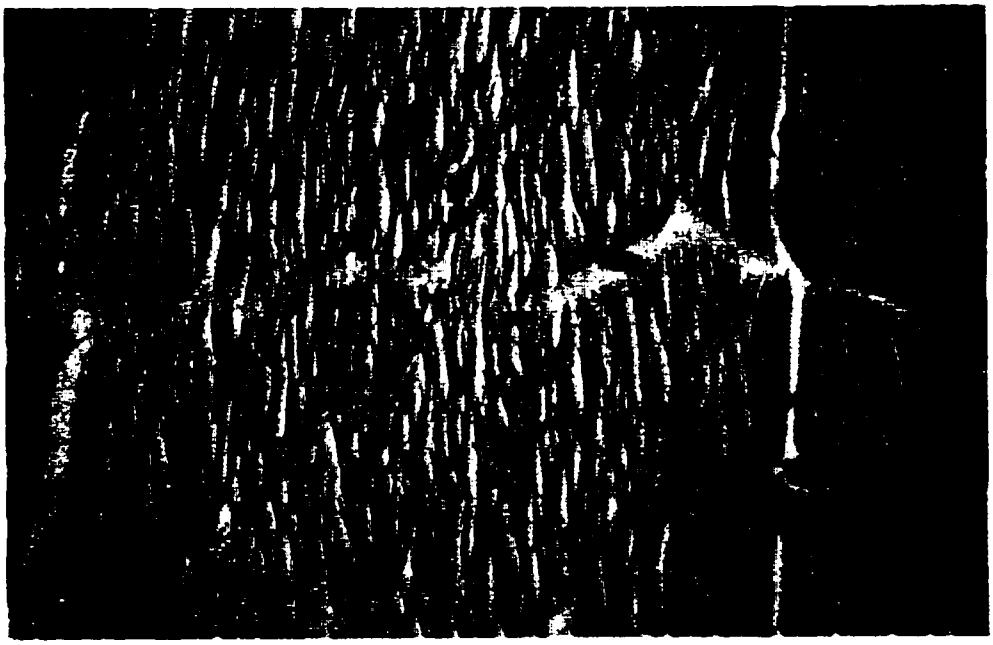
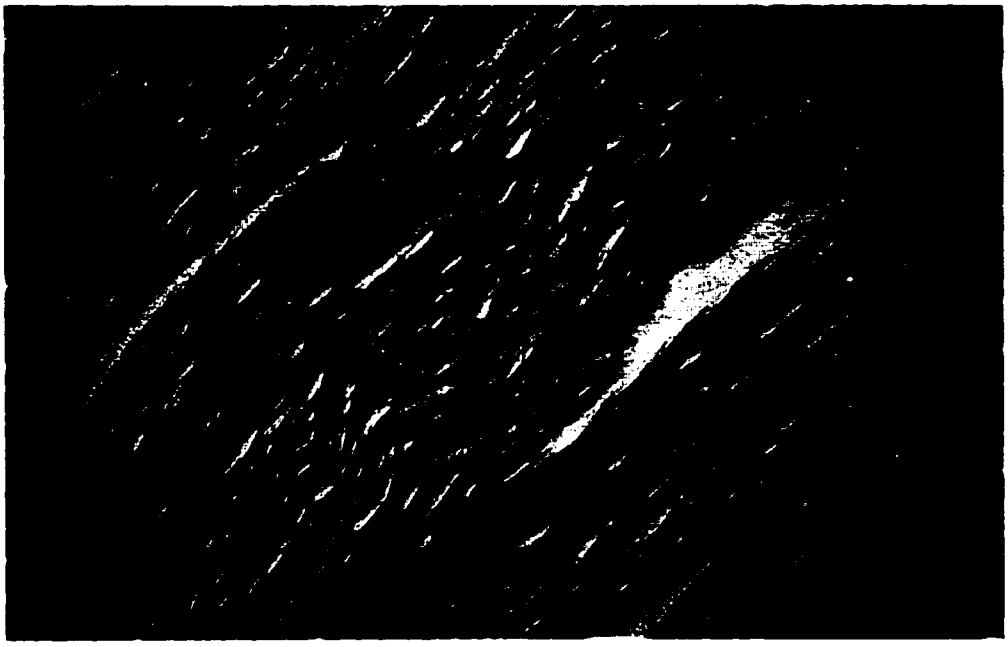


FIGURE 21. HISTOLOGY OF SOLEUS MUSCLE. Hematoxylin and eosin stained 4μm sections of soleus muscle from UCP-3Tg (top) and control (bottom) mice fed a normal chow diet. Magnification x 400.

Soleus Muscle Histology

UCP-3Tg



Control

FIGURE 22. HISTOLOGY OF GASTROCNEMIUS MUSCLE. Hematoxylin and eosin stained 4μm sections of gastrocnemius muscle from UCP-3Tg (top) and control (bottom) mice fed a normal chow diet. Magnification x 400.

Gastrocnemius Muscle Histology

UCP-3Tg

Control

3.3 Studies with a Distinct Group of Women in the Ottawa Hospital Weight Management Program

3.3.1 Baseline Characteristics of Diet-responsive and Diet-resistant Weight Loss Cohorts and for Biopsied Subgroups

The baseline characteristics of biopsied subjects are demonstrated in Table 1. There were no significant differences between the cohort group and the biopsied subjects, or the diet-resistant and diet-responsive subjects in terms of age, initial body weight, initial BMI or age of onset of obesity. Thus, our biopsied subjects were representative of the larger population of subjects in each of the upper and lower quintiles for the rate of weight loss.

3.3.2 Weight changes in biopsied group

Despite similar baseline weight, age and BMI, mean weight loss after six weeks on a 900 kcal diet was 40% greater ($p=0.0005$) in the diet-responsive as compared to the diet-resistant individuals (Table 2). In addition, the percentage of initial body weight lost over the six week period was 51% higher in the diet-responsive compared to the diet-resistant subjects. At the time of the biopsy, there was no significant difference ($p=0.72$) in body weight or weight loss between the diet- resistant or -responsive individuals (Table 2). The mean number of months from completion of the Weight Loss Program to the muscle biopsy of the diet-responsive individuals was 22.1 mo ($\pm$ 4.1) and 18.7 mo ($\pm$5.0) for the diet-resistant individuals.

TABLE 1:

BASELINE CARACTERISTICS OF DIET-RESPONSIVE AND DIET-RESISTANT WEIGHT LOSS COHORTS AND FOR BIOPSIED SUBGROUPS

	BIOPSIED SUBJECTS		COHORT	
	Diet-responsive (n=12)	Diet-resistant (n=12)	Diet-responsive (n=71)	Diet-resistant (n=70)
Age at program entry (yr)	44.0 (±1.67)	45.2 (±3.38)	41.07 (±0.96)	45.07 (±1.96)
Initial BMI (kg/m^2)	37.07 (±1.50)	41.0 (±2.54)	37.39 (±0.58)	40.61 (±1.2)
Initial Weight	218.50 (±9.39)	235.02 (±14.7)	218.0 (±4.10)	240.4 (±6.47)
Average # of parents overweight	1.17 (±0.17)	1.41 (±0.15)	1.16 (±0.09)	1.14 (±0.10)
% overweight with onset after age 20yr	50%	33.3%	28%	31.9%

TABLE 2:

WEIGHT CHANGES IN THE BIOPSIED SUBGROUPS

	WEIGHT (lb)		WEIGHT CHANGE (lb)	
	Diet-responsive	Diet-resistant	Diet-responsive	Diet-resistant
At baseline	218.5 (±9.38)	235.0 (±14.7)		
After 6wks meal replacement	191.3 (±8.26)	216.0 (±13.25)	**27.1 (±1.20)**	**19.3 (±1.49)**
At biopsy	184.8 (±9.53)	192.4 (±19.1)	33.7 (±6.99)	42.6 (±6.99)

* Results shown as mean (± SEM)

3.3.3 Skeletal Muscle UCP3 mRNA Abundance in Diet-responsive and Diet-resistant Women

Ucp3 mRNA abundance in skeletal muscle was determined to be 25% greater in diet-responsive versus diet-resistant biopsied subjects ($p<0.001$) (Figure 23). However, mRNA levels were not significantly correlated with either age, weight or BMI at the time of study.

3.3.4 Overall Kinetics of Skeletal Muscle Mitochondrial Proton Leak Reaction in Diet-responsive and Diet-resistant Individuals

Studies with isolated mitochondria from diet-responsive and diet-resistant subjects revealed that the overall kinetics of mitochondrial proton leak reactions are significantly different between the two groups (Figure 24). Mitochondrial oxygen consumption at any given value of protonmotive force is higher for diet-responsive subjects than in diet-resistant individuals. For example, at 150 mV, leak-dependent oxygen consumption is approximately 36% higher in diet-responsive individuals than in diet-resistant subjects. State 4 respiration was determined to be 51% higher in mitochondria from diet-responsive individuals than in those isolated from diet-resistant subjects (183.3 ±14.0 versus 121.1 ±15.0 nmol$O \cdot min^{-1} \cdot$ mg $protein^{-1}$). State 4 Δp values, however, were not significantly different between the two groups.

The equations for the lines of best fit were generated as described in the Methods section. R^2 values for goodness of fit were 0.9985 and 0.9049 for diet-responsive and diet-resistant subjects, respectively.

FIGURE 23. SKELETAL MUSCLE UCP3 MRNA ABUNDANCE IN DIET-RESPONSIVE VERSUS DIET-RESISTANT SUBJECTS. Results shown represent means ±SEM of (p=0.006).

Relative UCP3 mRNA Abundance

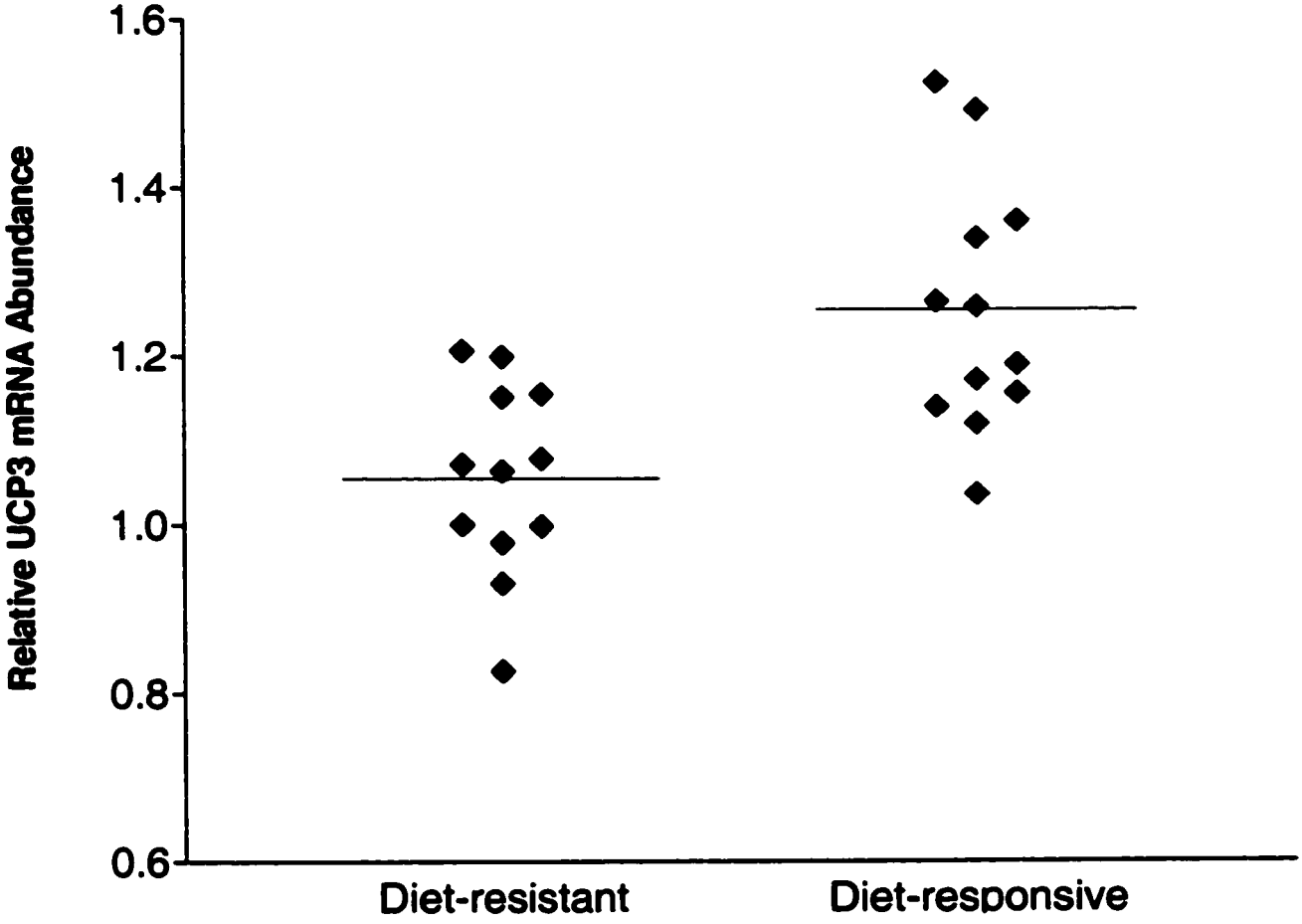
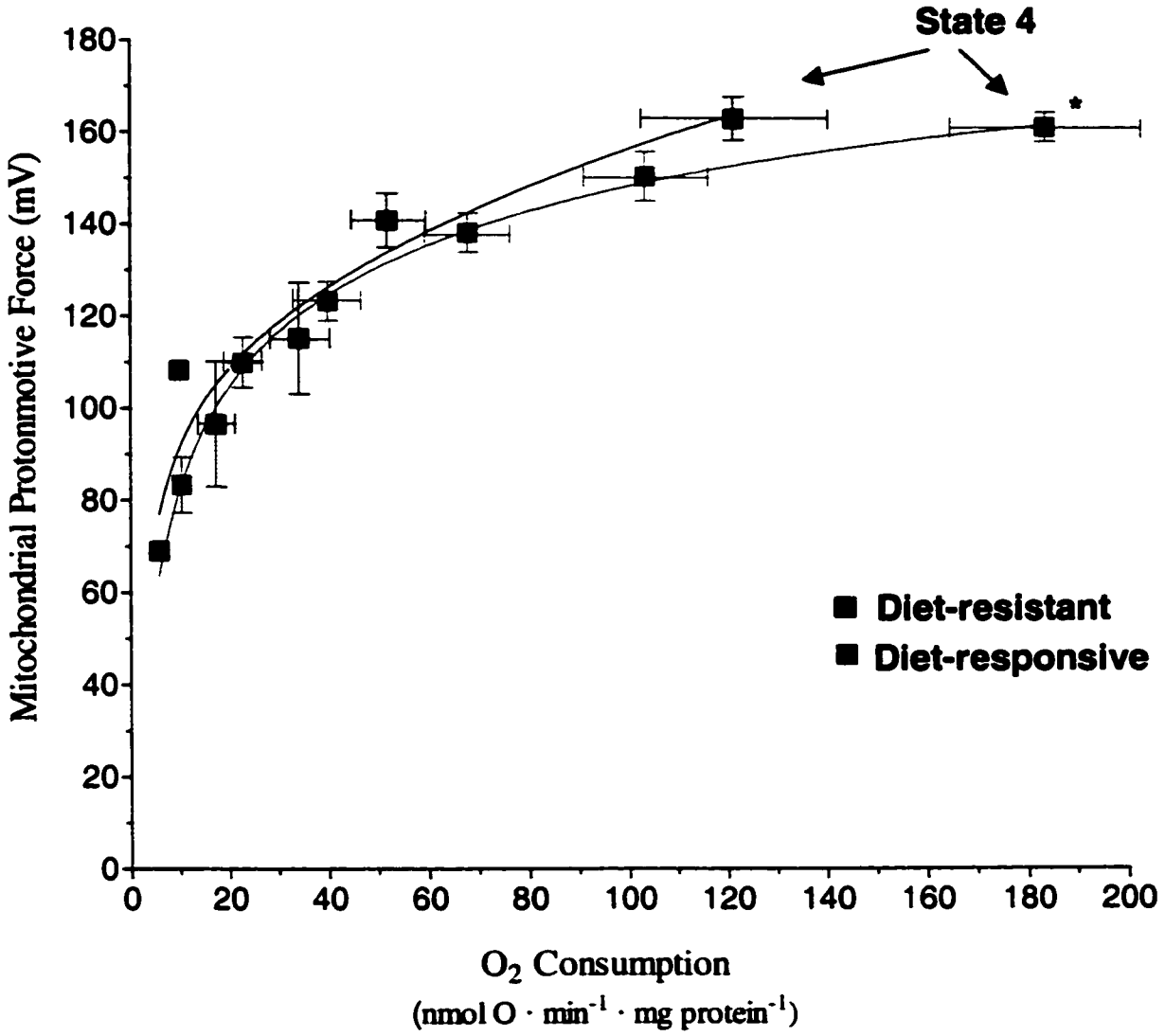


FIGURE 24. OVERALL KINETICS OF SKELETAL MUSCLE MITOCHONDRIAL PROTON LEAK REACTIONS IN DIET-RESPONSIVE AND DIET-RESISTANT SUBJECTS. The kinetic response of the proton leak to Δp was determined by titration of state 4 (maximal, non-phosphorylating) respiration with increasing amounts of a complex II inhibitor, malonate (0.66, 1.0, 2.0, 3.0 and 5.0 mM) in the presence of saturating amounts of oligomycin (8 ug/mg mitochondrial protein). Each point represents the mean ±SEM of duplicate experiments with mitochondria from 12 diet-responsive and 12 diet-resistant individuals. * denotes a statistically significant difference in *oxygen consumption* between the two groups (p= 0.0062).

Skeletal Muscle Mitochondrial proton leak Kinetics of Obese Diet-responsive vs. Diet-resistant Women

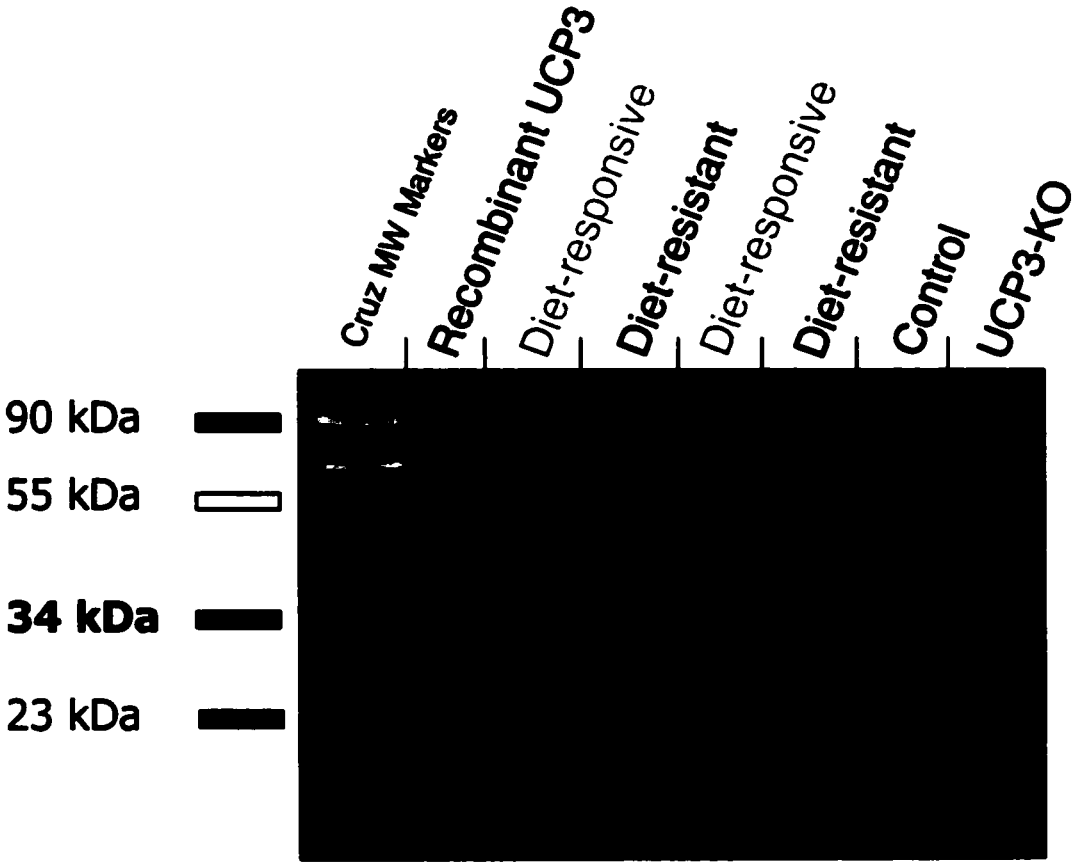


3.3.5 Western Blotting

Immunoblotting did not reveal any significant differences between diet-responsive and diet-resistant UCP3 protein levels (Figure 25). Due to the small quantity of mitochondrial protein remaining after proton leak assessments, it was difficult to perform a sufficient number of Western blots to determine unequivocally whether or not differences exist.

FIGURE 25. WESTERN BLOTS OF SKELETAL MUSCLE MITOCHONDRIAL PREPARATIONS FROM DIET-RESPONSIVE AND DIET-RESISTANT SUBJECTS. The location and size of biotinylated molecular weight markers (lane 1) correspond to those of Rainbow™ molecular weight markers depicted on the left of the blot. Lanes 2 contains recombinant UCP3, lanes 3 and 5 contain skeletal muscle mitochondria from *diet-responsive* individuals, and 4 and 6 both contain skeletal muscle mitochondria from *diet-resistant* subjects. Lane 7 contains skeletal muscle mitochondria from wild-type control mice and lane 8 contains skeletal muscle mitochondria from *Ucp3*-knockout mice. The primary antibody used was a polyclonal rabbit anti-human UCP3; the secondary antibody was an anti-rabbit IgG horseradish peroxidase conjugate. Exposure time was 30 minutes.

Western Blot



4. DISCUSSION

It has long been known that UCP1 is responsible for facultative energy expenditure in rodent BAT. Upon stimulation by the sympathetic nervous system (SNS), UCP1 allows protons to return to the mitochondrial matrix, bypassing ATP synthesis. The resulting dissipation of the electrochemical gradient allows substrate oxidation to occur without concomitant capture of some of the potential energy via the formation of ATP. The net effect during activation of UCP1 (by diet or cold) is that substrate oxidation is uncoupled from phosphorylation with a resultant increase in thermogenesis. The absence of significant amounts of this tissue in adult humans, and the desire to reveal the mechanisms modulating skeletal muscle thermogenesis, led to the search for other proteins homologous to UCP1 which could also uncouple oxidative phosphorylation.

The ongoing search for other Ucp genes in tissues other than BAT finally led to the discovery of the Ucp2 (Fleury *et al.*, 1997) and Ucp3 genes (Boss *et al.*, 1997b; Vidal-Puig *et al.*, 1997), as well as Ucp4 and brain mitochondrial carrier protein-1 (BMCP1) genes (Mao *et al.*, 1999; Sanchis *et al.*, 1999). UCP3 is of particular interest as a potential mediator of thermogenesis because it is selectively expressed at moderately high levels in human skeletal muscle (Boss *et al.*, 1997b; Vidal-Puig *et al.*, 1997), a tissue that contributes significantly to resting energy expenditure (Rolfe and Brown, 1997). Thus far, the *assumption* has been that, by analogy to UCP1, the homologues of UCP1 mediate thermogenesis by uncoupling oxidative phosphorylation. This has been supported by in vitro studies which have demonstrated that transfection or overexpression of Ucp3 in yeast or other cell culture systems results in increased mitochondrial uncoupling (Ricquier and Bouillaud, 2000).

However, more recent investigations into the physiological function of UCP1 homologues have resulted in findings which challenge the hypothesis that UCP2 and UCP3 are indeed uncoupling oxidative phosphorylation.

In light of this debate over the function of UCP1 homologues, we made it the central objective of my thesis to try to elucidate the true physiological role of the UCP1 homologues, and of UCP3 specifically. We did this by examining mitochondrial proton leak and UCP3 protein expression in: (1) mice that had been transgenically altered such that they do not express Ucp3, (2) transgenic mice that overexpress human Ucp3 in their skeletal muscle, (3) and finally in a distinct population of women who differ in the rate of weight loss. The question we, and numerous others, are trying to answer is: Is UCP3 functioning as an mitochondrial uncoupler, or does it serve a predominantly different purpose?

Our studies with *Ucp3*-knockout mice show that the mitochondrial proton conductance is reduced in skeletal muscle when UCP3 is absent (Gong *et al.*, 2000) (Figure 7). This result supported our hypothesis that UCP3 is a significant contributor to proton leak in skeletal muscle, and that the remaining UCPs in muscle (*e.g.*, UCP2, or other yet to be discovered proteins) do not compensate for the loss of UCP3. It is also important to point out that we detected an increase in body weight and adiposity in the *Ucp3*-knockouts as compared to controls. We originally hypothesized that perhaps the reason for this difference was the reduced proton conductance in the absence of UCP3. However, Dr. Reitman's group at the NIDDK,NIH did not observe any differences in body weight or adiposity in studies of much larger groups of *Ucp3*-knockouts and controls. The reason for our incongruent results could be due to the fact that we studied only a subset of early generations

(F1) of these mice of hybrid genetic background and the NIH group extended studies of larger numbers of mice into later generations of backcrosses onto more pure genetic backgrounds. Nevertheless, the decreased leak we observed in our mice was consistent with the increased adiposity in these mice at the time we studied them.

In our studies with the *Ucp3*-knockouts, we also examined the effects of various free fatty acids on proton leak kinetics in isolated skeletal muscle mitochondria. Our results demonstrate that in control mitochondria (*i.e.*, in the presence of UCP3), linoleic acid (f.c. 27 μM) and lauric acid (f.c. 66 μM) both increase state 4 (leak dependent) oxygen consumption and decrease state 4 protonmotive force (*i.e.*, increase proton leak), while stearic acid (27 μM) had no significant effect (Figure 10-12). In the absence of UCP3, none of the fatty acids had an effect on the kinetics of the leak. These results are consistent with those of Klingenberg *et al* (Klingenberg and Huang, 1999), Garlid *et al* (Jaburek *et al.*, 1999), and Hoffmann *et al* (Hofmann *et al.*, 2001) who have shown that lauric acid (C12:0) and linoleic acid (C18:2(n-6)) (Klingenberg and Huang, 1999) are potent stimulators of UCP1-mediated proton transport.

For our second set of studies we decided to focus on transgenic mice that overexpress human Ucp3 in skeletal muscle (UCP-3Tg mice). UCP-3Tg mice have total Ucp3 mRNA expression that is 66-fold greater than normal (Clapham *et al.*, 2000), and despite an increased food intake, they are more lean than controls. By examining the overall kinetics of skeletal muscle mitochondrial proton leak reactions, we have shown that proton conductance is significantly increased in mitochondria from UCP-3Tg mice (Figure 15). Our data, and the more limited findings on mitochondrial energetics originally published by Clapham and

coworkers (Clapham *et al.*, 2000), are all consistent with a role for UCP3 in energy expenditure. The results presented herein provide evidence that UCP3 under these conditions (very high level of expression) may indeed be functioning as an uncoupler of oxidative phosphorylation and a conductor of protons, and it might therefore influence energy balance in vivo. In the absence of this protein we measured a decrease in proton leak, and when it was overexpressed we observed the opposite. These results are in line with studies which show that yeast transfected with Ucp2 or Ucp3 have decreased mitochondrial membrane potential, supporting the hypothesis that these homologues uncouple oxidative phosphorylation. However, it can be argued that the level of Ucp3 overexpression is non-physiological and our results need to be reconciled with a number of paradoxes in the literature and with other studies that support a different role for UCP3.

One piece of evidence that suggests the lack of a role for UCP3 in thermogenesis is indicated by the cold-intolerance of the *Ucp1*-knockout mouse, in which UCP3 does not substitute for UCP1 in cold-induced non-shivering thermogenesis (Enerbäck *et al.*, 1997). In addition, contrary to expectations, fasting, a condition that is known to reduce muscular energy expenditure and increase energy efficiency in humans and animals (Gavrilova *et al.*, 1999; Grande *et al.*, 1958; Ma and Foster, 1986; Samec *et al.*, 1998b; Trayhurn and Jennings, 1988) has been shown to ***increase*** Ucp3 expression in muscle (Boss *et al.*, 1998a; Boss *et al.*, 1997a; Boss *et al.*, 1998c; Cadenas *et al.*, 1999; Ricquier and Bouillaud, 2000; Weigle *et al.*, 1998). Weigle and colleagues have shown a 10-fold increase in Ucp3 mRNA expression in rat quadricep muscle between 12 and 24 h of food deprivation (Weigle *et al.*, 1998), and more recently, Cadenas and coworkers demonstrated that starvation increased Ucp2 and

Ucp3 mRNA levels more than 5-fold and 4-fold, respectively, and UCP3 protein levels by 2-fold (Cadenas *et al.*, 1999). What was very interesting in the latter study, an observation which is contradictory to our data, is that despite a significant increase in UCP2 and UCP3 in the starved rat muscle, proton conductance remained unchanged; this suggests that these UCPs expressed at physiological levels may not mediate the basal proton conductance in skeletal muscle mitochondria. The results from starvation experiments present a major challenge for the hypothesis that UCP3 is involved in increasing thermogenesis; if the physiological role of UCP3 is energy expenditure, and energy expenditure is decreased during a fasted state, then why does the level of UCP3 increase? One possibility is that perhaps the activity of UCP3 is used for purposes other than thermogenesis.

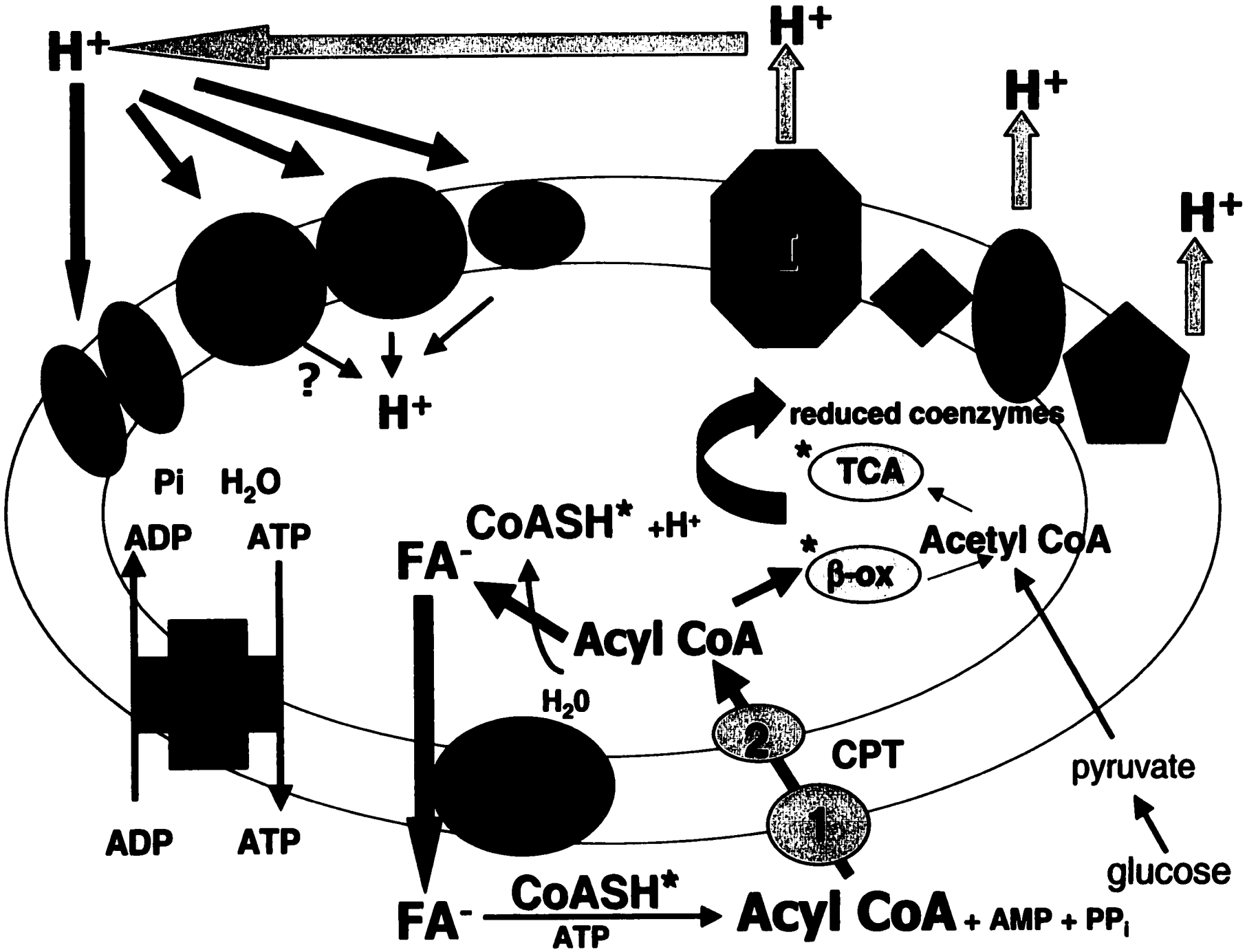
In the last few years more studies have been emerging which show a very strong correlation between Ucp3 expression and free fatty acid levels. In addition to fasting, acute thyroid stimulation (Gong *et al.*, 1997), exercise (Boss *et al.*, 1998b; Tsuboyama-Kasaoka *et al.*, 1998), and administration of a lipid emulsion (Khalfallah *et al.*, 2000; Weigle *et al.*, 1998) are all conditions identified as increasing free fatty acid levels as well as increasing Ucp3 expression. Interestingly, reports also show that newborn mice do not express Ucp3 in skeletal muscle until ingesting a fat-rich meal (*i.e.*, suckling) (Brun *et al.*, 1999). In shivering skeletal muscle there is a transient increase in Ucp3 mRNA between 3 and 24 hours of cold exposure, followed by a return to normal levels, and then a reduction to lower than normal levels after about 10 days (Boss *et al.*, 1998c; Larkin *et al.*, 1997; Lin *et al.*, 1998). These changes are correlated with a transient rise in plasma free fatty acid levels in response to cold. Situations in which fatty acid levels are decreased, such as hypothyroidism (Gong

et al., 1997) and chronic starvation (Boss *et al.*, 1998c), have also been correlated with a ***decrease*** in Ucp3 mRNA expression. Thus, the expression of Ucp3 mRNA in muscle seems to be linked to a switch to oxidation of fatty acids in this tissue, whether energy expenditure is increased or decreased. Another piece of evidence that makes this idea more plausible is the fact that fasting-induced upregulation of Ucp3 still occurs in rats at thermoneutrality (29°C) (Samec *et al.*, 1998b). This refutes the idea that the increase in Ucp3 mRNA during food deprivation is to meet thermoregulatory needs.

The findings from studies which correlate free fatty acid levels with Ucp3 mRNA expression are thus much more consistent with a role for UCP3 in the regulation of fatty acid metabolism than in thermogenesis and energy balance. The idea that the role of UCP3 is the regulation of fatty acid metabolism was originally suggested by Samec and colleagues (Samec *et al.*, 1998b), and has slowly become more accepted by the research community. What remains to be elucidated is the exact mechanism by which UCP3 does this. A mechanism to explain how UCP3 might enhance fatty acid oxidation has recently been proposed by Himms-Hagen and Harper (Himms-Hagen and Harper, 2001). They postulate that perhaps the role of UCP3 in fatty acid oxidation is that of exporting fatty acid anions (one of the confirmed capabilities of UCP1, UCP2 and UCP3; (Jaburek *et al.*, 1999)) from the mitochondrial matrix, promoting rapid rates of fatty acid oxidation.

Briefly, during fatty acid oxidation, plasma free fatty acids enter the muscle and are activated to acyl-CoA in the cytosol, a reaction that requires CoASH and energy in the form of ATP (Figure 26). The acyl group is then transferred to carnitine by CPT1 (carnitine palmitoyl transferase 1) at the outer surface of the mitochondrial inner membrane. After

FIGURE 26. OUTLINE OF MITOCHONDRIAL FATTY ACIDMETABOLISM, PROTON LEAKS, AND THE HYPOTHESIZED ROLE OF UCP3 IN EXPORT OF FATTY ACID ANIONS IN MUSCLE AND BAT MITOCHONDRIA. (Adapted from Himms-Hagen and Harper, 2001)


H+
H+
H+
H+
H+
?
reduced coenzymes
*
TCA
Pi
H2O
ADP
ATP
CoASH* +H+
FA-
Acetyl CoA
*
β-ox
Acyl CoA
H2O
2
CPT
pyruvate
1
ADP
ATP
glucose
FA- CoASH* / ATP Acyl CoA + AMP + PPi

being shuttled to the mitochondrial matrix, the acyl group is transferred back to CoA by CPT2 and the carnitine returns across the inner membrane to the cytosol. Acyl-CoA then enters the β-oxidation pathway, which converts acyl-CoA to acetyl CoA. The acetyl CoA produced in the β-oxidation cycle is oxidized in the tricarboxylic acid cycle (TCA cycle), with enzymes on the inside of the inner membrane or in the matrix. The reduced coenzymes NADH and $FADH_2$ are then oxidized in the electron transport system, entering at Complex I or Complex II, respectively. NADH+ H^+ is produced per β-oxidation cycle, by pyruvate hydrogenase and by three reactions of the TCA cycle (at isocitrate dehydrogenase, ketoglutarate dehydrogenase, and malate dehydrogenase steps). $FADH_2$ is produced in the β-oxidation pathway by acyl-CoA dehydrogenase and by succinate dehydrogenase in the TCA cycle. As these coenzymes are oxidized at the inner membrane, energy is used by the various complexes to pump protons out of the mitochondrial matrix. The proton gradient that is subsequently produced drives the synthesis of ATP by the proton-translocating ATP synthase. The role of UCP3 in all of this, according to Himms-Hagen and Harper, is that it acts as an overflow route for fatty acid anions when acyl-CoA entry is in excess of its removal by oxidation. Since mitochondria do not import fatty acid anions, nor are they a byproduct of any metabolic pathway in the mitochondria, the only source of fatty anion would be from the hydrolysis of acyl-CoA by mitochondrial acyl-CoA thioesterases (MTE).

The enzymatic activity of MTEs is to cleave acyl-CoA long chain fatty acids to their corresponding free fatty acids and CoASH within the mitochondrial matrix, but its physiological function was not defined until recently. Expression of MTE mRNA is highest in tissues with high levels of β-oxidation (*e.g.*, skeletal muscle, BAT, WAT), and its mRNA

levels are regulated by PPARα (Hunt *et al.*, 1999; Svensson *et al.*, 1998), a transcription factor involved in fatty acid metabolism. Hence, it seems plausible that MTEs function as regulators of fatty acid oxidation. There exists a number of forms of MTEs, and recently, two new forms of this protein have been cloned (Poupon *et al.*, 1999; Svensson *et al.*, 1998), thus acyl-CoA can by hydrolysed by a number of MTEs in the matrix. The hydrolysis of acyl-CoA is physiologically beneficial since it regenerates steady supply of CoASH, which is needed for reactions involved in fatty acid oxidation (Figure 26) and is in high demand at times of dependence on fatty acid oxidation. As well, the liberated fatty acid anion can be transported back across the inner membrane by UCP3, returning to the pool of fatty acid anions in the cytosol, thus clearing the mitochondria of a molecule that it is potentially deleterious and it is unable to be metabolized. The energy used in this process is due to an ATPase effect rather than uncoupling due to proton return. Each fatty acid anion cycled through the protein results in the hydrolysis of two ATP molecules and liberation of one proton in the matrix, thus the more fatty acids cycled, the more energy expended.

Some more evidence for the idea that UCP3 functions as a fatty acid anion transporter was recently published by Moore *et al* who studied the expression of genes related to fatty acid metabolism in muscle such as fatty acid transporters (FAT (CD36) and FATP), lipoprotein lipase (LPL), fatty acid binding protein (FABP and H-FABP), carnitine palmitoyl transferase (CPT-Iβ), and MTE-1 (Moore *et al.*, 2001) in UCP-3Tg mice. Mice overexpressing Ucp3 demonstrated a 3-fold increase in MTE-1. The only other mRNA that was significantly increased was LPL (50%). Recently, the same research group tried to determine whether situations which cause changes in Ucp3 mRNA levels also cause

concordant changes in MTE-1 mRNA. They did this by examining Ucp1, Ucp2, Ucp3 and MTE-1 mRNA levels in various tissues of *db/db* and *db/+* mice. Indeed, when there were changes in Ucp3 mRNA, there was a complimentary change in MTE-1 message, suggesting that MTE-1 and UCP3 are involved in the same pathway either in response to, or as regulators of fatty acid oxidation. The expression of other enzymes involved in fatty acid metabolism has been recently studied in human muscle by Hildebrandt and Neufer (Hildebrandt and Neufer, 2000). They have shown that increases in Ucp3 message resulting from a 24 hr fast also increases the levels of message of enzymes involved in fatty acid metabolism such as LPL, CPT I, LCAD (long-chain acyl-CoA dehydrogenase). In another study, Kratky and coworkers have shown that Ucp3 mRNA levels are induced 3.4-fold in mice with high levels of LPL in skeletal muscle, and down-regulated in mice that lack LPL (the presence of absence of LPL in BAT had no effect on Ucp3 expression levels) (Kratky *et al.*, 2001). All these studies validate the hypothesis that UCP3 is likely involved in fatty acid metabolism during times of increased fatty acid oxidation.

Studies have also been conducted to examine the extent to which the metabolic challenges imposed by acute fasting influence the transcriptional regulation of genes important for metabolic control in skeletal muscle in different muscle fibre types. Samec and coworkers have shown that the degree of upregulation of Ucp3 as a result of fasting is more pronounced in type IIb muscle fibres (fast-twitch glycolytic (FG); gastrocnemius and tibialis anterior) than type I muscle fibres (slow-twitch oxidative (SO); soleus) (Samec *et al.*, 1998a). This muscle-type dependency is closely linked to increases in CPT-1 and medium-chain acyl-CoA dehydrogenase (MCAD). The addition of nicotinic acid (anti-lipolytic agent) did not blunt

the transcription of these enzymes, suggesting that perhaps the surge in fatty acids upon fasting may not be a major afferent signal that initiates the events leading to the upregulation of the UCPs and enzymes regulating lipid oxidation. In order to explore the physiological role of UCP3 in human skeletal muscle, Hesselink *et al* studied the expression of UCP3 in distinct human muscle fibre types in vastus lateralis muscle (Hesselink *et al.*, 2001). They found that type I fibres had the lowest expression of Ucp3 mRNA, while type II fibres had the highest expression. Upon subclassification of the type II fibres into type IIa (fast twitch oxidative-glycolytic (FOG) and type IIb (FG), it was shown that the IIb fibres had the highest levels of protein. This study is in accordance with observations on the Ucp3 message levels in whole muscle homogenates in selected rat muscles (Boss *et al.*, 1997b; Samec *et al.*, 1998a). The observation that the changes in UCP2 and UCP3 expression during fasting are more pronounced in predominantly glycolytic muscles than in predominantly oxidative is consistent with the greater dependency of oxidative muscles on lipids as fuel substrate, and the greater shift between glucose and lipid as fuel substrate in glycolytic muscles during starvation. In other words, under conditions in which lipids are the primary fuel substrates (and glucose availability is limited), UCP3 and fatty acid metabolic enzymes are upregulated so as to serve as an alternative route for fatty acid metabolism and spare glucose.

It is possible to reconcile our findings from studies of the UCP-3Tg mouse with the hypothesis that UCP3 is not functioning as an uncoupler, but a transporter of fatty acids. Despite increased food consumption these mice are more lean, had an increased clearance rate for glucose, and had absolute levels of plasma triglycerides and non-esterified fatty acids that were comparable to controls. Circulating levels of non-esterified fatty acids and triglycerides

reflect a balance between release into, and uptake from, the circulation (Moore *et al.*, 2001). It is conceivable that upon overexpression of UCP3, the protein acts to increase both uptake and release, and keep plasma levels of both of these molecules normal. The observed increase in mitochondrial proton conductance and increase in respiration rates can also be explained by an increase in efflux of fatty acid anions via UCP3. The energy used is due to an ATPase effect rather than uncoupling due to proton return. Each fatty acid anion cycled through the protein results in the hydrolysis of two ATP molecules and liberation of one proton in the matrix (Himms-Hagen and Harper, 2001), thus the more fatty acids cycled, the more energy expended. Observed decreases in mitochondrial protonmotive force could be due to the fact that when a fatty acid anion leaves the matrix and enters the cytosol, the electrochemical potential is reduced. This would explain why, for any given oxygen consumption value, protonmotive force was lower in UCP-3Tg mitochondria compared to controls. Our results are contrary to those of Cadenas *et al* who did not observe any changes in proton conductance when UCP3 protein levels were increase 2-fold (Cadenas *et al.*, 1999). This could be due to the fact that we were trying to detect difference in proton conductance when Ucp3 mRNA levels are expressed at levels 66 times higher than normal, and they were studying mice with only a 4-fold increase in Ucp3 mRNA, an amount that may not produce detectable metabolic changes. Another recent study by Cadenas *et al* suggests that UCP3 is not responsible for the basal proton conductance of skeletal muscle mitochondria, although it can catalyse an inducible proton conductance when activated by superoxide (in the presence of fatty acids) (Cadenas *et al.*, 2001). They draw this conclusion from the fact that they did not observe a superoxide-induced increase in proton conductance in skeletal muscle

mitochondria of mice overexpressing Ucp3, an assay which they suggest is indicative of native UCP3 function analagous to that for UCP1. However, the physiological significance of this hypothesis is not clear. Regardless of the mechanism of action of UCP3, all of the data suggest that overexpression of hUcp3 in mice plays a significant role in the resistance to obesity and the development of diabetes. The fact that enzymes which are involved in the regulation of fatty acid β-oxidation (*i.e.*, MTE-1) are increased in the UCP-3Tg mouse supports the idea that perhaps the phenotype observed in these mice is a result of UCP3 facilitating fuel oxidation by exporting fatty acids when oxidation rates are increased. However, this remains to be established experimentally.

Although our results indicate increased body weight and adiposity in the *Ucp3*-knockout, this was not confirmed in studies using larger numbers these mice. Later generations of *Ucp3*-knockout mice show no significant signs of obesity, normal thermoregulation, and normal responses to thyroid hormone and fasting (Gong *et al.*, 2000). We showed that muscle mitochondria of the knockouts have a higher membrane potential but no change in state 4 oxygen consumption (Figure 7), suggesting no decrease in mitochondrial proton leak but some change in their bioenergetic process. Looking at our data from the point of view that UCP3 may be acting as a fatty acid anion efflux protein, then perhaps the reason for higher membrane potentials in the absence of UCP3 is due to fewer fatty acid anions being transported out of the matrix which would decrease potential.

Our results showing increased skeletal muscle mitochondrial oxygen consumption and decreased membrane potential in the presence of linoleic and lauric acids in wild-type mitochondria, but not in *Ucp3*-knockout mitochondria (Figure 10,11), confirm that fatty acid

effects are mediated by UCP3. However, the mechanism by which fatty acids are entering the mitochondria and mediating their effects, is not yet known. A recent study by Hamilton and Kamp (Hamilton and Kamp, 1999) suggests that neutral non-esterified fatty acids (NEFAs) can enter the mitochondria by free diffusion through the phospholipid bilayer, and then flip-flop to the matrix side of the membrane. Then, as the neutral fatty acids enter the matrix they are deprotonated due to a proton gradient across the inner mitochondrial membrane, resulting in a lowering of the proton gradient (*i.e.*, uncoupling). Further evidence that the role of UCP3 lies in fatty acid metabolism comes from a very recent study conducted by Bézaire and colleagues. They show that fasting in *Ucp3*-knockout and wild-type mice results in significant differences in their respiratory quotients, suggesting impaired fatty acid oxidation in the absence of UCP3 (Bézaire *et al.*, 2001).

In addition to rodent studies, we, and others, have been investigating Ucp3 expression and its role in human metabolism. As previously mentioned, human genes for Ucp2 and Ucp3 have been mapped to human chromosome 11q13, with a separation of only 7kb between the two genes (Fleury *et al.*, 1997; Solanes *et al.*, 1997). A linkage of markers in the vicinity of these genes with resting metabolic rate, percentage body fat and fat mass, and hyperinsulinemia has been reported (Bouchard *et al.*, 1997). A positive correlation has been reported between hUcp3 mRNA in muscle, hUcp2 mRNA in adipose tissue, and components of metabolic rate (reviewed by Langin *et al* (Langin *et al.*, 1999)). During a 5 hour triglyceride infusion, increased plasma nonesterified fatty acids levels induced hUcp3 (but not hUcp2) expression in muscle, but physiological hyperinsulinemia appears to prevent this induction of Ucp3 mRNA (Khalfallah *et al.*, 2000). Studies with Type II diabetics have

shown that mRNA levels of hUcp3, but not hUcp2, are significantly reduced in skeletal muscle compared to controls (Krook *et al.*, 1998) and fasting does not increase these levels (Vidal *et al.*, 1999). Recently, Schrauwen and colleagues have examined the effect of a 10 week weight reduction program (~ 500 kcal/day for the first 4 wks, then ~ 1000 kcal/day for 4 wks) on skeletal muscle Ucp2 and Ucp3 mRNA expression and UCP3 protein content in Type II diabetic subjects (Schrauwen *et al.*, 2000). They found that Ucp3 mRNA decreased from pre-diet measurements, and the changes in mRNA and protein were negatively associated with changes in body weight and body mass index (BMI). They suggest that the decrease in UCP3 during weight loss could be occurring as a metabolic adaptation to prevent further weight loss. Changes in Ucp3 mRNA and protein levels induced by diet were positively correlated with changes in cytosolic fatty acid binding proteins, implying again a role for UCPs in the handling of lipids as a fuel.

Several genetic variants of Ucp3, as well as Ucp1 and Ucp2 have been investigated for their contribution to the development of obesity and/or diabetes (see Table 3). Notably, a single nucleotide polymorphism (SNP) in the 5 ′ flanking region of the hUCP3 gene, -55 C ➡ T, has shown to be associated with elevated hUcp3 expression in male non-diabetic Pima Indians (a population in South Western USA that is susceptible to obesity), but Otabe *et al* (Otabe *et al.*, 1999; Otabe *et al.*, 1998) found no association between the polymorphism and obesity in French Caucasians who were normoglycemic or diabetic and morbidly obese. A recent study by the same group has shown that the C ➡ T polymorphism may be associated with BMI in interaction with physical activity (Otabe *et al.*, 2000). Recently, Lanouette *et al* found strong associations between a polymorphism in the sixth intron on

Ucp3 (GAIVS6) and BMI, fat mass, percentage body fat, and leptin levels (Lanouette *et al.*, 2001). The fact that the GAIVS6 increases adiposity lends support to the hypothesis that UCP3 plays a role in regulating adiposity. Notably, Argyropoulos and coworkers have also found a missense SNP in exon 3 (V1021), a terminal polymorphism in the exon 6-splice donor region (IVS6 + 1G>A), and a mutation introducing a stop codon in exon 4 (R143X) in two severely obese Gullah-speaking African American probands, but not in Caucasians (Argyropolous *et al.*, 1998). In the exon 6-splice donor heterozygotes, basal fat oxidation rates were reduced by 50%, and their respiratory quotient was increased compared to normal individuals, suggesting a role for UCP3 in fatty acid oxidation and metabolic fuel partitioning (Argyropolous *et al.*, 1998). However, Chung *et al* did not observe the same association between respiratory quotient and the exon 6-splice donor mutation in African Americans from Maywood, Illinois (Chung *et al.*, 1999). This discrepancy could be due to the fact that the Maywood group have a higher level of European admixture levels than the Gullah African Americans(*i.e.*, a different genetic background) (Argyropoulos and Harper 2001, In Press).

The overall goal of our human study was to determine metabolic, genetic and biochemical determinants of weight loss in response to an energy restricted diet. More precisely, our objective was to examine the role of UCP3 and mitochondrial proton leak in patients on an Optifast™ program who were identified as either *diet-responsive or diet-resistant* individuals. Statistical analyses have revealed that initial weight and age explained 30% of the variability in weight loss and 35% of the variability in body fat loss. Initial serum free T_3 concentration could only explain an additional 5% of the weight loss. Thus, two-thirds of the variability in weight loss was left unexplained by factors known to regulate

energy requirements. The hypothesis is that genetically based differences in Ucp3, and differences in mitochondrial energetics could determine efficiency of energy substrate conversion to cellular ATP and hence explain the variability in an individual's ability to lose weight.

Muscle biopsies (~ 2-3 g) were taken from the *rectus femoris* (mixed fibre types) region of the quadricep of matched pairs of diet-responsive and diet-resistant subjects, mitochondria were isolated and mitochondrial proton leak determinations were carried out. Our pooled results from 11 diet-resistant and 11 diet-responsive subjects indicate that the state 4 oxygen consumption is 51% higher in the diet-responsive subjects compared to the diet-resistant ones (Figure 24). This increase in oxygen consumption, however, is not associated with differences in state 4 protonmotive force. Immunoblotting mitochondrial samples from our study subjects has not shown any detectable difference in the level of UCP3 protein between the two groups (Figure 25). However, as discussed earlier, there were only limited amounts of mitochondria. Thus our findings must be regarded as equivocal. Regardless of actual protein levels in the diet-responsive and diet-resistant groups, the regulation of the activity of this protein could be quite different in the two groups. Determination of muscle Ucp3 mRNA levels was conducted by André Gauthier, a graduate student in Dr. McPherson's lab in the Ottawa Heart Institute. Ucp3 mRNA expression was found to be 25% greater in skeletal muscle of diet-responsive compared to diet-resistant individuals, suggesting that the upregulation of this protein could be responsible for the differences seen in the rate of weight loss. Dr. Tesson, another member of our extended research group, has been conducting an extensive genetic screening of key 5' flanking region

transcriptional regulatory sequences in the subjects biopsied. Since thyroid hormones are the only known endocrine regulators of leak, we screened for genetic variation in the thyroid response element (TRE). However, we did not find any variation in the TRE. Also in this region, the three putative peroxisome proliferator response elements, PPRE1 (-1677 to -1690), PPRE2 (-456 to -468), and PPRE3 (-242 to -254) were screened for variation by PCR/sequencing in the subjects studied. However, we did not detect any polymorphic variation in any of these response elements. Diet-responsive and diet-resistant obese women were also screened for all previously reported polymorphisms and mutations in the humanUcp3 gene (See Table 3). They did not reveal any association between a particular genotype that may account for the differences in the ability to lose weight. However, the results from these studies were obtained from a limited number of patients (*i.e.*, 24) and therefore may not reflect the influence of genotype on the ability to lose weight among these women.

The observed differences in leak between these two distinct groups of individuals, and the increase in Ucp3 mRNA in the diet-responsive group, suggests that mitochondrial energetics in human muscle mitochondria may be a potential regulator/predictor of energy metabolism and thus result in one's ability to gain/lose weight. However, further studies with a larger human population are indeed warranted. However, given the great invasiveness of the procedures required for proton leak analyses (*i.e.*, 2-3 g muscle biopsy), it would be advantageous to develop other less invasive approaches for the study of proton leak in muscle of humans.

TABLE 3:

GENETIC VARIANTS IN THE HUMAN UCPS

	Genetic Variant	Type of SNP	Reference
hUcp1	-112A>T	5' UTR	(Mori *et al.*, 2001)
	Met229Leu	missense	(Mori *et al.*, 2001)
	-3826A>G	promoter	(Cassard-Doulcier *et al.*, 1996)
	X>Y	5' UTR	(Urhammer *et al.*, 1997b)
	W>Z	5' UTR	(Urhammer *et al.*, 1997b)
	Arg40Trp	missense	(Urhammer *et al.*, 1997b)
	Ala64Thr	missense	(Urhammer *et al.*, 1997b)
	Val137Met	missense	(Urhammer *et al.*, 1997b)
	Lys257Asn	missense	(Urhammer *et al.*, 1997b)
hUcp2	Ala55Val	missense	(Urhammer *et al.*, 1997a)
	-2723T>A	promoter	(Esterbauer *et al.*, 2001)
	-1957G>A	promoter	(Esterbauer *et al.*, 2001)
	-866G>A	promoter	(Esterbauer *et al.*, 2001)
	-371G>C	promoter	(Esterbauer *et al.*, 2001)
	13nt-del/insl	promoter	(Esterbauer *et al.*, 2001)
hUcp3	-439InsA	promoter	(Otabe *et al.*, 2000)
	-155TC>T	promoter	(Otabe *et al.*, 2000)
	-55C>T	promoter	(Schrauwen *et al.*, 1999)
	+5G>A	5' UTR	(Otabe *et al.*, 2000)
	Val9Met	missense	(Otabe *et al.*, 1999)
	Arg70Trp	missense	(Brown *et al.*, 1999)
	Ala83Ala	silent	(Otabe *et al.*, 1999)
	Tyr99Tyr	silent	(Otabe *et al.*, 1999)
	Val102Ile	missense	(Argyropolous *et al.*, 1998)
	Arg143*	stop codon	(Argyropolous *et al.*, 1998)
	Tyr210Tyr	silent	(Otabe *et al.*, 1999)
	Arg308Trp	missense	(Otabe *et al.*, 1999)
	Intron4C>T	intronic	(Otabe *et al.*, 1999)
	IVS6+ 1G>A	exon 6 splice	(Argyropolous *et al.*, 1998)

5. SUMMARY AND CONCLUSIONS

Skeletal muscle mitochondria of *Ucp3*-knockout mice have higher membrane potential but no change in respiration, suggesting that UCP3 may not be a proton translocating protein. The absence of any effect of fatty acids in mitochondria lacking UCP3, but a significant effect in control mitochondria, indicates that UCP3 is required for mediating the fatty acids effects we have observed. While results from studies of Ucp3-knockouts and Ucp3 overexpressors have overall been useful, there are, however, possible pitfalls in interpreting results from gene knockout and transgenic studies. The lack of a distinct phenotype in these mice could be a result of the planned genetic outcome, as well as the secondary responses of the organism to the genetic modification (*i.e.*, compensatory mechanisms) (Williams andWagner, 2000). The fact that mice lacking UCP3 show other biochemical impairments in skeletal muscle metabolism such as increased reactive oxygen species production (Vidal-Puig *et al.*, 2000) and a tendency for impaired starvation-induced shift in lipid partitioning between oxidation and storage (Muoio *et al.*, 2000), suggests that perhaps these impairments in muscle metabolism must have been compensated for by other mechanisms. Thus, it is quite possible that the failure of UCP-knockout mice to reveal impairments in energy expenditure, weight regulation, and substrate metabolism is due to compensatory mechanisms and not a direct result of gene knockout. In contrast, mice overexpressing human Ucp3 showed markedly elevated metabolic rates, lipid oxidation and increased proton leak. Whether the primary effect the Ucp3 overexpression is an increase in heat production as a result of exaggerated UCP3-mediated proton leak, or an increase in UCP3-mediated fatty acid flux across the mitochondria with resulting uncoupling effects, is not known. It must be noted that the

massive upregulation of Ucp3 mRNA expression (>66-fold) in the muscle of these mice may result in increased protein synthesis to an extent that could lead to impaired mitochondrial membrane integrity. This could account for the increase in proton leak observed (*i.e.*, artifactual uncoupling). The possible drawbacks of using gene knockout and transgenic mice aside, our results overall show that UCP3 is somehow playing a role in muscle mitochondrial metabolism. As well, our studies have shed some light on the possible role of UCP1 homologues in mitochondria, albeit a role that may not involve mediating adaptive thermogenesis.

Our results from human studies have provided the first ever measurements of proton leak in human muscle mitochondria. The observed differences in leak between individuals who are diet-responsive of diet-resistant suggests that mitochondrial energetics in human muscle mitochondria may be a potential regulator of energy metabolism during negative energy balance. It remains to be decided whether the physiological function of UCP3 is primarily associated with uncoupling or with energy substrate partitioning and fatty acid metabolism. Regardless of the mechanistic details, our findings are consistent with an important role for mitochondrial proton leak and UCP3 in regulating the efficiency of energy metabolism. UCP3 and proton leak may thus be candidates for pharmacological upregulation of fatty acid oxidation in obese, diet-resistant subjects.

Given the mounting evidence, it seems unlikely that UCP3 is involved in mediating thermogenesis or basal proton conductance in mitochondria. Physiological changes which are known to alter adaptive thermogenesis such as fasting and overfeeding do not result in changes in basal proton conductance. Fasting, a state of energy conservation, results in

increased levels of Ucp2 and Ucp3 mRNA, demonstrating that the role of these proteins is probably not that of energy expenditure. In contrast, there are strong associations between the UCP2 and UCP3 and the regulation of lipids as fuel substrate in skeletal muscle, and strong links between UCP2 and the control of reactive oxygen species production. At this point in time, it appears as though UCP3 acts in concert with UCP2 in the overall regulation of lipid oxidation concomitant to the prevention of lipid-induced oxidative damage. Of course, more studies are required before the exact mechanism of UCP3 and its potential role in the regulation of lipids as fuel substrate can be elucidated.

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B.Sc. (Hon.)	University of Ottawa, Faculty of Science, 1996 *Department of Biochemistry*
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TEACHING AND RESEARCH EXPERIENCE

Jan-Mar 2001 **University Lecturer**
University of Ottawa, Faculty of Science
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1999- present **Ph.D. candidate,**
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1996-present **Teaching Assistant/Laboratory Demonstrator**
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1999- present **M.Sc. candidate,**
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Summer 1996 **Research Assistant**
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ACADEMIC AND RESEARCH AWARDS

2000	**Ontario Graduate Scholarship** • Research Grant ($12, 000)
2000	**University of Ottawa Excellence Scholarship** • Full coverage of tuition expenses (~ $5,000)
1999	**Ontario Graduate Scholarship** • Research Grant ($12, 000)
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1999	**University of Ottawa Ph.D. Entrance Award**
1998	**Eva Princz Memorial Award** • Travel grant awarded to attend International Congress on Obesity conference (Paris, France)
1998	**Young Investigator of the Year Award** *Awarded to the best research presentation* • North American Association for the Study of Obesity Annual Meeting; Paris, France
1997	**Young Investigator of the Year Award FINALIST** • North American Association for the Study of Obesity Annual Meeting; Cancun, Mexico
1992	**Ontario Scholar** Lisgar Collegiate Institute

VOLUNTEER EXPERIENCE

July 2000-May 2001	**Canadian Society for International Health** Trans Caucus Health Information Program
1999-present	**Crisis Line Counsellor** Ottawa Rape Crisis Centre
1997-1998	**Junior Girls Basketball Coach** Gloucester-Cumberland Basketball Association
1997-present	**University of Ottawa Graduate Students' Association** Co-President and Treasurer
Summer 1993/94	**Children's Hospital of Eastern Ontario** Emergency room and ICU volunteer

ADDITIONAL SKILLS

- Experience as research group team leader and mentor to junior researchers
- Excellent presentation, verbal, oral, and teaching capabilities (see awards)
- Extensive experience with all Microsoft Office, Corel Office, and statistical analysis software

LANGUAGES

- English, French, Farsi (Persian)

INTERESTS AND ACTIVITIES

- National Capital Outdoors Club
- Photography (expeditions to Ecuador and the Middle East)
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- Violin

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RESEARCH PUBLICATIONS

Gong D-W, **Monemdjou S**, Gavrilova O, Harper M-E, and Reitman ML (2000) Normal response to fasting and thyroid hormone and lack of obesity in mice lacking uncoupling protein-3.
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